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THE MULTIPLICATION OF FIBROBLASTS IN VITRO.

By ALEXIS CARREL, M.D., and ALBERT H. EBELING, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 14, 1921.)

It is well known that the life of tissues cultivated in the plasma of an adult animal lasts only for a certain period of time. 10 years ago, one of the writers attempted to prevent the death of the tissues living under these conditions.[1] It was supposed that if their waste products were removed by frequent washings, the cells would multiply indefinitely. Every few days, fragments of embryonic heart, cultivated in plasma, were washed in Ringer solution, and placed in a fresh medium. The duration of their life was very much increased, but death still occurred sooner or later. No tissue could be kept alive for more than 3 months.[2] It was evident that the adult plasma did not contain the substances necessary for the indefinite multiplication of fibroblasts. This fact was confirmed by Quagliariello,[3] who injected serum proteins into dogs, and found that they are not used as food by the tissue cells. In the more recent experiments of Kerr, Hurwitz, and Whipple,[4] there was no evidence that serum proteins may be considered as intermediary products between food proteins and parenchyma proteins. Although connective tissue cannot live indefinitely in plasma, it often displays a great activity for several days or weeks. What is the origin of the substances used by the cells in the process of multiplication? The opinion of Lewis,[5] and Ingebrigtsen[6] is that the tissues grow within the limits determined by the amount of food stored up in the body of each individual cell. Burrows[7] also believes that the food material of the tissues is not derived from the medium but from the cells within the fragment. The growth observed in the culture would be a simple transfer of material from the cells of the center of the fragment to those which have migrated into the medium. When growth ceases after a few transplanta-

[1] Carrel, A., *J. Am. Med. Assn.*, 1911, lvii, 1611.

[2] Carrel, A., *J. Exp. Med.*, 1912, xv, 516.

[3] Quagliariello, G., *Arch. fisiol.*, 1912, x, 150.

[4] Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918–19, xlvii, 356.

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277.

[6] Ingebrigtsen, R., *J. Exp. Med.*, 1912, xvi, 421.

[7] Burrows, M. T., *Anat. Rec.*, 1916–17, xi, 335. Burrows, M. T., and Neymann, C. A., *J. Exp. Med.*, 1917, xxv, 93.

tions, the sum of the total growth would be less than the original mass. Although no accurate measurements have been made, there is no doubt that a fragment of tissue cultivated in the plasma of an adult animal does not increase materially in size, in spite of the active proliferation of the cells. But the cause of this temporary activity has not as yet been ascertained.

It is also well known that the addition of embryonic tissue juice to the plasma of an adult animal activates the rate of cell division and brings about an immense increase in the mass of the tissue.[8] A strain of fibroblasts derived from a small fragment of embryonic heart has produced about 30,000 cultures in the past 9 years, and is as active today as at the beginning of its life. If this strain had been allowed to grow freely, the volume of tissue so produced would be very much larger than the earth. There is no doubt, therefore, that when embryonic juice is added to adult plasma new cells are made from the substances contained in the medium, and that this process can go on indefinitely.[9]

It then appears that adult plasma alone, and plasma mixed with embryonic juice, differ widely in their action on the growth of tissue. The reason for these differences is still incompletely known. It is certain that a mixture of embryonic juice and adult plasma had the power to increase the rate of cell division. But the part played respectively by the constituents of the medium in the multiplication of the cells is not thoroughly understood. The purpose of this article is to investigate whence come the substances used by the fibroblasts cultivated in adult plasma alone, and what constituents of the medium are responsible for the increase of the mass of the tissue when embryonic juices are added to the plasma.

I.

Although the mass of a tissue cultivated in the plasma of an adult animal does not increase, or increases very little, the building of the new cells requires some material which must come from the plasma or from the tissue itself. In order to determine the part played by the plasma, the action of its constituents, fibrin and serum, was separately studied. Connective tissue having been found to grow as extensively in fibrin fixed by formaldehyde as in normal fibrin, the rôle of fibrin must be considered as purely mechanical. In a first series of experi-

[8] Carrel, A., *J. Exp. Med.*, 1913, xvii, 14. Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273. Carrel, A., *J. Exp. Med.*, 1913, xviii, 287; 1914, xx, 1.

[9] Ebeling, A. H., *J. Exp. Med.*, 1919, xxx, 531.

ments, the influence of serum was investigated by measuring the growth of connective tissue in media containing no serum, and serum of different concentrations. In a second series of experiments, the possible action of substances contained in the tissues themselves was examined.

1. Rate of Growth of Tissue in Media Containing Varied Dilutions of Serum.—Fragments of embryonic heart and of a 9 year old strain of fibroblasts were cultivated in media composed of fibrinogen suspension and of Tyrode solution, and containing no serum, or serum in varied dilutions. The serum was taken from the plasma of chickens which had fasted 24 hours. The animals were about 2 years old and in good health. The serum was preserved in paraffined or Pyrex tubes, and its hydrogen ion concentration was measured. Sometimes it was found slightly modified after a few days. The serum was diluted with Tyrode solution sterilized by filtration through a Berkefeld filter. The suspension of fibrinogen was prepared by a technique already known.[10] A medium composed of 10 per cent fibrinogen suspension and 90 per cent Tyrode solution gave a firm coagulum in which embryonic heart tissue could be cultivated without the occurrence of liquefaction. But if a fragment of old strain of fibroblasts was used, liquefaction took place. A little serum had to be added to the medium to prevent this accident. The other media were made of 10 per cent fibrinogen suspension and of mixtures of Tyrode solution and of serum, such that the concentration of serum varied from 2.37 to 90 per cent. The cultures were prepared and measured according to a method previously described.[11] The area of the original fragment was measured immediately after the preparation of the culture, and 48 hours later. The growth was expressed generally by the relative increase of the surface of the tissue; that is, the total area minus the area of the original fragment, divided by the area of the original fragment. The ratio of the relative increase of the experiment to the relative increase of the control permitted the comparison of the growth of cultures belonging to different experiments or groups of experiments. The absolute increase of the tissue was known by the

[10] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiii, 641.

[11] Ebeling, A. H., *J. Exp. Med.*, 1921, xxxiv, 231.

width of the ring of new tissue around the original fragment, measured with a micrometer.

In ten experiments, embryonic heart tissue was cultivated in media containing from 0 to 90 per cent serum (Table I). The growth of every fragment in the experimental medium was compared to the growth of a control in plasma. The increase of the tissues was expressed by the width of the ring of fibroblasts. The experiments in which the layer of new tissue was of unequal thickness were discarded. The figures of Table I show that the tissues grew at least as well in Tyrode solution containing no serum as in plasma. The growth was larger in media, the serum concentration of which varied from 2.37 to 85 per cent, than in plasma. When the amount of growth obtained in a given concentration of serum was compared with that obtained, not in plasma, but in another concentration of serum, it was found that the action of a medium containing 2.37 per cent serum was as marked as that of a medium containing 90 per cent serum. The growth of embryonic heart appeared to be independent of the concentration of the serum as long as the amount of fibrin was not modified. This shows that the cells in their process of multiplication do not make use of the serum to an extent measurable by the present method.

In thirty experiments, an old strain of fibroblasts was used instead of embryonic heart (Table II). It did not grow in a medium composed only of fibrin and Tyrode solution, because the coagulum liquefied in a few hours. There was practically no difference between the amount of growth of the fragments cultivated in media the serum concentrations of which varied from 2.37 to 80 per cent. When fragments of the old strain were cultivated in 10 per cent serum and 50 per cent serum, and kept in the same media for several passages, no difference in the amount of new tissue was observed, even after four passages, as is shown by Text-fig. 1. In several experiments, two fragments of the old strain were studied comparatively in plasma and in 10 per cent serum. The tissues grew at about the same rate, in spite of the great difference in the composition of the medium. The rate of growth decreased rapidly and in about the same way in both media. Death occurred after the sixth or seventh passage. In Text-fig. 2 is shown a typical experiment. The decrease in the rate of growth is more rapid during the first passages in normal plasma

TABLE I.

Growth of Embryonic Heart Tissue in Plasma and Varied Dilutions of Serum, Expressed in Terms of Width of Ring.

Experiment No.	Culture No.	Composition of medium.																				Remarks.
		Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	
			0 per cent.		2.37 per cent.		8.3 per cent.		16.5 per cent.		20.5 per cent.		22 per cent.		30 per cent.		33 per cent.		50 per cent.		85 per cent.	
1	18585				1.5		1.8		1.1													
2	18656	0.2	0.4	0.1	0.2	0.2	0.1	0.05	0.2	0.2	1.0					0.2	2.0	0.1	2.0			
3	18700	0.5	Liquefied.	0.4	1.6	0.3	2.0	0.2	0.3													
4	18735	0.6	1.0	0.1	1.6	1.0	1.0	0.7	1.6			0.7	1.3	0.6	1.6							
5	18795	0.8	1.0	0.8	1.1	0.7	1.3	0.8	1.2			0.8	1.2	1.0	1.4							
6	18840	0.5	0.5	0.5	2.0	0.4	1.6	0.7	1.8			0.7	1.5	0.6	0.5							
7	18864	1.5	2.0	1.5	4.0	1.6	4.5	1.3	3.3			1.1	3.5	1.1	1.1							
8	18916	0.8	2.0	0.6	2.2	0.5	3.6	0.6	1.2			0.6	1.1	0.8	1.6					0.9	1.2	
9	18951							0.7	0.5													
10	18962	0.9	2.5	1.2	1.2	1.9	1.7															90 per cent serum control instead of plasma.
Average.		0.6	1.0	0.6	1.7	0.8	1.9	0.6	1.2	0.2	1.0	0.8	1.7	0.8	1.2	0.2	2.0	0.1	2.0	0.9	1.2	

The increase of the tissues is expressed by the width of the ring of fibroblasts which invaded the medium over a period of 48 hours. In the first nine experiments, the control was cultivated in adult plasma. In the tenth experiment, the control was cultivated in 90 per cent serum instead of plasma.

TABLE II.

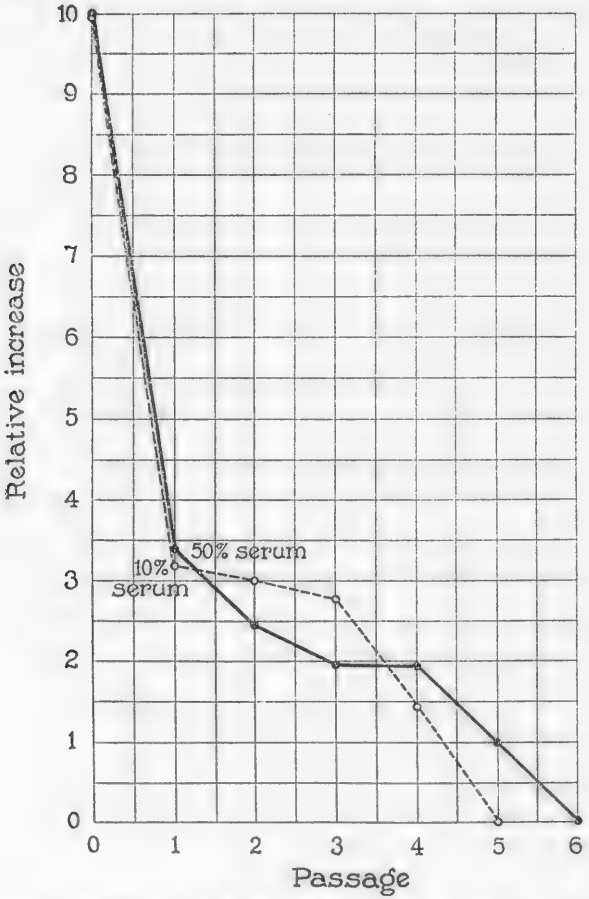
Growth of an Old Strain of Fibroblasts in Plasma and Varied Dilutions of Serum.

Experiment No.	Culture No.	Date.	Composition of medium.													
			Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, plasma.	Experiment, serum.	Control, serum.	Experiment, serum.	Control, serum.	Experiment, serum.	Control, serum.	Experiment, serum.	Control, serum.	Experiment, serum.
				2.37 per cent.		8.3 per cent.		16.5 per cent.	90 per cent.	22 per cent.	90 per cent.	30 per cent.	10 per cent.	50 per cent.	10 per cent.	80 per cent.
		1920														
1	18651	Dec. 13			1.0	1.2										
2	18684	" 20	0.1	0.2	(After 24 hrs.)											
3	18784	" 20	2.5	1.5												
4	18785	" 20	2.0	1.2												
5	18786	" 20			2.2	2.2										
6	18787	" 20			1.6	1.8										
7	18861	" 23	1.7	1.5												
8	18862	" 23	Average,	Average,	1.7	1.5										
9	18863	" 23	2.07	1.6	1.5	1.5										
10	18951	" 28			Average,	Average,	1.1	1.2								
		1921			1.6	1.64										
11	19046	Jan. 3							0.5	0.9						
12	19047	" 3							0.7	1.0						
13	19048	" 3							0.6	0.8						

14	19049	Jan. 3	Control serum.								0.8	0.9				
15	19050	" 3	90 per cent.								0.75	1.0				
16	19051	" 3									0.7	0.8				
17	19067	" 5	Few cells.	1.6	Control serum.											
18	19068	" 5	" "	1.6	91 per cent.											
19	19069	" 5	" "	1.9												
20	19098	" 8		Average,	0.1	1.5										
21	19233	" 17		1.7	0.5	1.1										
22	19234	" 17			0.6	1.0										
23	19235	" 17			0.4	1.3										
24	19237	" 17			0.5	1.3										
25	19364	" 28			Average,	Average,										
26	19365	" 28			0.42	1.22										
27	19366	" 28														
28	19385	" 30											2.0	2.0	1.0	1.0
29	19386	" 30											2.0	2.2	1.1	1.2
30	19387	" 30											2.2	2.2	1.0	1.2
Average							1.1	1.2	0.6	0.9	0.75	0.9	2.07	2.13	1.03	1.13

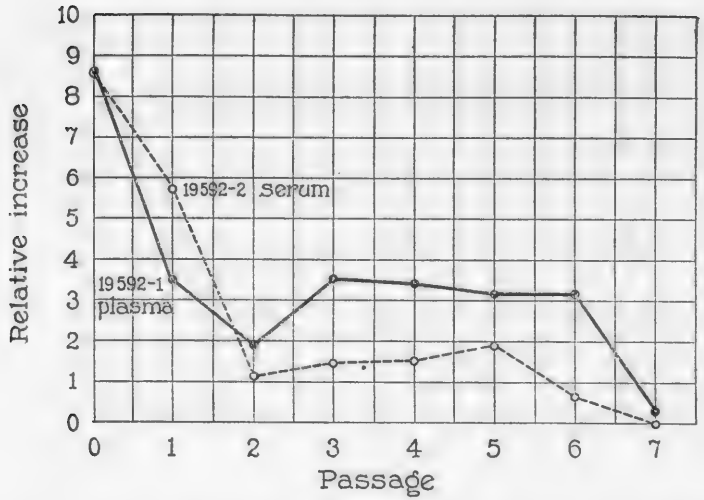
The increase of the tissue is expressed in terms of the width of the ring of new tissue produced in 48 hours.

than in a medium containing only 10 per cent serum. Afterwards, there are only small differences in the amount of tissue produced in both media, and death occurs at the same time. This completes the



TEXT-FIG. 1. Comparison of the rate of growth of two fragments of an old strain of fibroblasts in media containing 10 and 50 per cent serum, in the course of five and six passages (Experiment 19364).

demonstration that the fibroblasts in adult plasma or serum do not make use of the serum proteins.



TEXT-FIG. 2. Comparison of the rate of growth of an old strain of fibroblasts in adult plasma and 10 per cent serum, in the course of seven passages (Experiment 19592).

2. Rate of Growth of Tissue in Plasma.—Fragments of embryonic heart and of an old strain of fibroblasts were cultivated in adult plasma, transferred into new medium every 48 hours, and studied comparatively. The heart was taken from 10 to 14 day old chick embryos, cut in small fragments in a little Ringer solution, and imbedded in plasma. Coagulation always occurred spontaneously after a few minutes, and the growth was measured at the end of 48 hours. During several passages, the tissues displayed great activity, and the surface of the original fragment increased, as well as the width of the ring of new tissue. The rate of growth reached its maximum after a few passages. Then it progressively decreased and death occurred after from ten to thirty passages. The individual differences in the length of life of the cultures were probably due to the nature of the plasma and to the technique used in the transfer of the tissue. The details of two typical experiments are given as an illustration in Table III and Text-fig. 3. The surface of the original fragment, the surface of the new tissue, and the relative increment of the fragment at

each passage were calculated. In the first experiment, the value of the relative increment increased as far as the fourth passage. At the same time, the area of the fragment became larger and reached its maximum at the fifth passage. Later, it progressively decreased and death occurred after the twelfth passage. In the second experiment there was also an increase in the rate of growth of the fragment and

TABLE III.

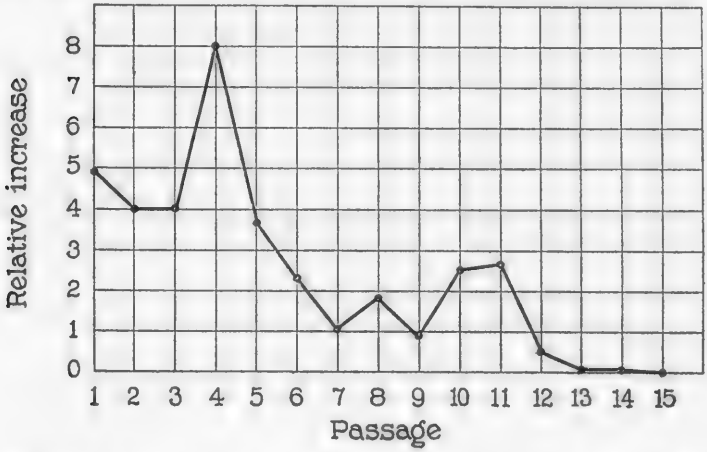
Growth of Embryonic Heart Tissue in Adult Plasma during Fifteen and Eleven Passages (Experiments 18947 and 18948).

Experiment 1.						Experiment 2.					
Passage No.	Culture. No.	Date.	Area of fragment.	Area of growth.	Relative increase.	Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.
		1920						*1920*			
1	18947	Dec. 29	1.6	9.1	5.69	1	18948	Dec. 28	1.0	4.9	4.9
2	19006	" 30	1.8	5.7	3.2	2	19007	" 30	1.5	6.0	4.0
		1921						*1921*			
3	19028	Jan. 1	2.1	12.5	5.92	3	19029	Jan. 1	2.5	10.0	4.0
4	19044	" 3	2.5	19.3	7.7	4	19045	" 3	2.1	16.9	8.0
5	19065	" 5	5.1	20.1	4.0	5	19066	" 5	2.6	9.5	3.65
6	19091	" 7	3.5	11.5	3.85	6	19092	" 7	3.3	7.5	2.29
7	19122	" 10	4.4	4.3	1.0	7	19123	" 10	2.8	3.0	1.07
8	19152	" 12	2.7	2.8	1.0	8	19153	" 12	2.3	4.2	1.8
9	19189	" 14	1.2	0.7	0.06	9	19190	" 14	1.9	1.6	0.89
10	19214	" 15	1.5	5.2	3.45	10	19215	" 15	1.3	3.25	2.5
11	19231	" 17	2.1	Few cells.	0	11	19232	" 17	1.7	4.5	2.65
						12	19255	" 19	1.3	0.7	0.5
						13	19280	" 21	0.7	0.4	0.06
						14	19294	" 22	0.5	0.6	0.1
						15	19314	" 24	0.4	0	0

in their size, the maximum of which was reached at the sixth passage. It was impossible to know whether the active multiplication of the cells and the increase in the area of the original fragment during the first passages meant an increase in the volume of the fragment. This point could be ascertained if the tissues were weighed or the cells counted, but the precision of the method which was used is not sufficient to detect a slight and temporary increase of the mass. It

was obvious, however, that the activity of the cultures reached a maximum after a few passages, then decreased until death occurred. It seemed that in the beginning of its life *in vitro* the growth was activated by something which progressively disappeared.

Fragments of a 9 year old strain of fibroblasts were also grown in adult plasma. Previous to the experiment, the strain was kept in a condition of great activity in a mixture of plasma and embryonic juice. Its relative increase in 48 hours was generally from 6 to 10. The fragments were divided and put into plasma. Coagulation of



TEXT-FIG. 3. Growth of embryonic heart in adult plasma during fifteen passages (Experiment 18948).

the medium was obtained by a small piece of fibrin, because fibroblasts living *in vitro* cannot bring about the clotting of the plasma, as embryonic tissues do. Whenever the tissue was cultivated in adult plasma alone, the rate of growth became very much slower. According to the condition of the adult plasma, the decrease in the rate of growth and in the size of the fragment was more or less rapid. In several cases, death occurred after two or three passages, as shown in Table IV and Text-fig. 4. Before the experiment, the relative increase after 48 hours was 10.5. After the first, second, and third passages in adult plasma alone, the relative increase became respec-

tively 1.3, 0.8, and 0. In other experiments, the life of the old strain in adult plasma was longer, and death occurred after six or ten passages (Text-fig. 2). The variations were due to the degree of activity of the strain previous to the experiment, and to the condition of the plasma.

There was a striking difference between the growth of the fragment of embryonic heart and the strain of fibroblasts. As soon as the strain of fibroblasts was placed in plasma alone, the rate of multiplication of its cells decreased (Text-figs. 2 and 4), while under the same conditions the activity of a fragment of embryonic heart increased for several days (Text-fig. 3). The life of the heart was much longer than that of the strain of fibroblasts. These phenomena could not be

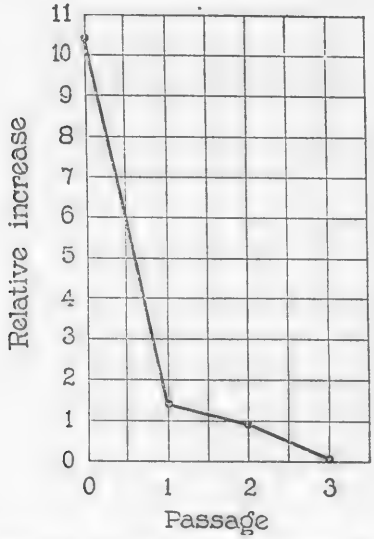
TABLE IV.

Growth of an Old Strain of Fibroblasts in Adult Plasma during Three Passages (Experiment 18907–2).

Passage No.	Culture No.	Date.	Area of fragment.	Area of growth.	Relative increase.
		1920			
1	18907–2	Dec. 26	5.7	59.3	10.4
	18947	" 28	5.0	6.8	1.36
		1921			
2	19006	Jan. 1	5.3	4.7	0.89
3	19028	" 3	5.3	0	0

attributed to a greater original activity of the embryonic heart, because the rate of proliferation of the fibroblasts of the 9 year old strain is at least as rapid as that of the connective tissue cells of a 10 or 12 day old embryonic heart. They might be due to substances contained in the tissue itself and more abundant in the embryonic tissue than in the strain of fibroblasts. It is known that tissue juices which activate the rate of cell division have also the power of coagulating fluid plasma. Chicken plasma is rapidly transformed into a solid coagulum by a small fragment of embryonic heart, even though the latter has been thoroughly washed in Ringer solution. On the contrary, a fragment of the old strain of fibroblasts does not bring about the coagulation of the plasma. It is, then, possible that embryonic heart contains some of the tissue juice which was found by

one of the writers to be capable of greatly accelerating the rate of cell multiplication,[1] while the strain of fibroblasts lacks it. During the first passages of embryonic heart, the small amount of juice contained in the tissue is probably responsible for the greater activity of the cells. When, after a few days, the original fragment has become surrounded by a dense reticulum of fibroblasts, the rate of growth de-



TEXT-FIG. 4. Growth of an old strain of fibroblasts in adult plasma alone during three passages (Experiment 18907–2).

creases just as it does in a culture of the old strain. It was observed frequently that if a cut were made through the original fragment, after growth had almost ceased, there was a resumption of activity, possibly due to the setting free of some embryonic juice. The activity displayed in adult plasma by both embryonic heart and old strain may be caused by the substances stored within the cells of the tissue, which have the power of increasing the rate of cell division, as is already known.

II.

The indefinite multiplication of fibroblasts, which occurs as soon as embryonic tissue juice is added to adult plasma, may be explained by two different hypotheses: the embryonic juice renders possible the utilization by the cells of substances contained in the serum or the fibrin; or it supplies itself the material required by the cells for their proliferation.

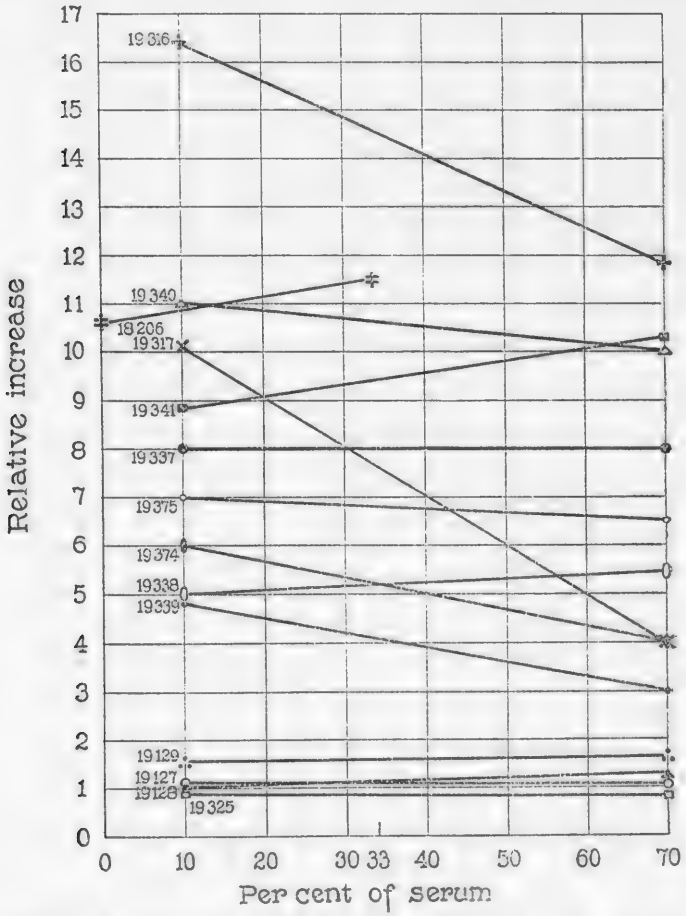
TABLE V.

Growth of an Old Strain of Fibroblasts in a Medium Composed of a Constant Amount of Tissue Juice and 10 and 70 Per Cent Serum.

Experiment No.	Culture No.	Date.	Relative increase.			
			Composition of medium.			
			Control.	Experiment.	Control.	Experiment.
			33 per cent.	0 per cent.	10 per cent.	70 per cent.
		1921				
1	18206		11.5	10.75		
2	19316	Jan. 24			16.4	11.8
3	19317	" 24			10.1	4.0
4	19325	" 25			0.9	0.8
5	19337	" 26			8.0	8.0
6	19338	" 26			5.0	5.4
7	19339	" 26			4.8	3.0
8	19340	" 26			11.0	10.0
9	19341	" 26			8.8	10.3
10	19127	" 10			1.1	1.0
11	19128	" 10			1.0	1.2
12	19129	" 10			1.6	1.6
13	19374	" 27			6.0	4.0
14	19375	" 27			7.0	6.5

Influence of Serum.—In a first group of experiments, it was attempted to find whether serum was used by the cells in the presence of embryonic juice. Tissue juices were obtained by centrifugation of a pulp made of 10 or 12 day old chick embryos. The media were composed of a constant amount of fibrinogen suspension and embryonic tissue juice, and of varied quantities of serum and Tyrode solution. Fragments of the 9 year old strain of fibroblasts were cultivated in these media. The coagula made of fibrin, embryonic juice, and Tyrode

solution liquefied after a few hours in all the experiments but one. In this experiment, the amount of new tissue was practically as large in



TEXT-FIG. 5. Growth of an old strain of fibroblasts in media containing a constant amount of tissue juice and varied dilutions of serum.

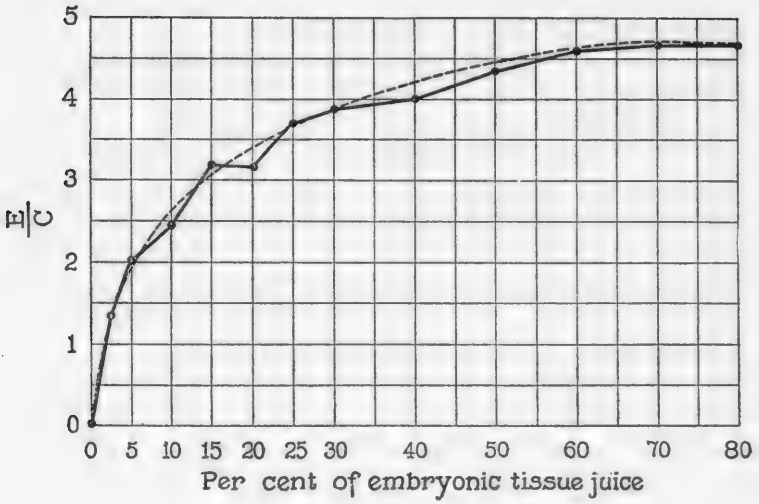
the medium containing no serum as in the medium containing 33 per cent serum. In thirteen experiments the media contained 10

and 70 per cent serum, and their action on the growing tissue was about identical, as is shown by the figures of Table V and by Text-fig. 5. The complete lack of serum, or its presence in low or high concentrations, had no influence on the rate of growth. This fact demonstrated that even in the presence of embryonic juices there was no utilization of the serum by the cells to a measurable extent.

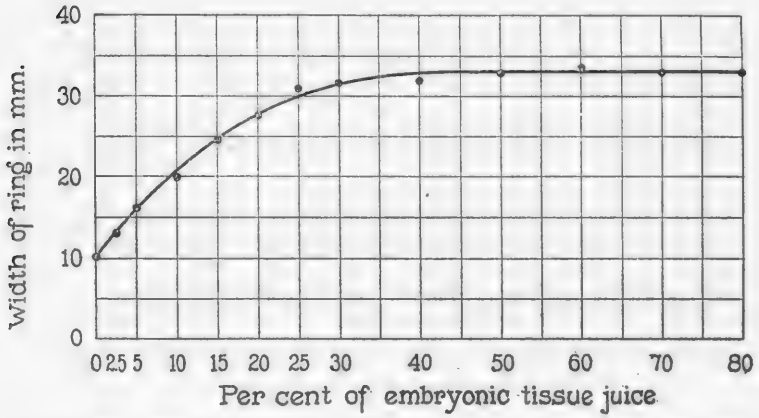
Influence of Fibrin.—In a second group of experiments, the action of the fibrin was studied. Plasma was coagulated by a little tissue extract at the surface of a cover-glass, and the coagulum was fixed in 2 per cent formaldehyde solution in Ringer solution for 1 hour. It became slightly bluish, but remained transparent. Then it was washed for 24 hours in distilled water, and for 24 hours in Ringer solution. It was then placed for half an hour in embryonic juice. The excess of embryonic juice was removed with blotting paper, and fragments of embryonic heart and of an old strain of fibroblasts were cultivated in the clot. The growth assumed the same characteristics and extent as in normal fibrin. In 48 hours, the width of the ring of reticulated fibroblasts was from 5 to 6, which is the same as in normal fibrin and embryonic juice. Since the growth in normal fibrin and in fibrin fixed by formaldehyde is the same, it is practically certain that fibrin plays only a mechanical rôle, and is not used by the cells.

Influence of Embryonic Juice.—In a third group of experiments, the embryonic juice was added to the medium in progressively increasing concentrations, while the amounts of fibrin and serum were kept constant. The tissues responded by considerable differences in the extent of growth, and these differences were the function of the concentration of the juice.

Fifty-five experiments were made. The action of the medium was ascertained by measurement of both the relative and the absolute increase of the tissue (Table VI). The ratios of relative increases of the experiments and of the control tissues were plotted in ordinates, while the concentrations of the juice present in the medium were plotted in abscissæ. The curve (Text-fig. 6) shows that the cells responded to the addition of small amounts of embryonic juice to the medium by a rapid increase of the rate of multiplication. Increases in concentration from 0 to 10 per cent brought about a greater change in the activity of the cells than increases from 10 to 80 per cent. After



TEXT-FIG. 6. Relation between the ratios $\left(\frac{E}{C}\right)$ of the relative increases of the tissue in experiments and controls, and the concentration of embryonic juice in the medium.



TEXT-FIG. 7. Relation between absolute increase of the tissue and the concentration of the embryonic juice in the medium.

TABLE VI.

Relation between the Amount of Growth Expressed in Terms of Relative Increase, Ratios of Relative Increases of Control and Experiments, and of Absolute Increase, and the Concentration of the Embryonic Juice in the Medium.

2.5 per cent tissue juice.						5 per cent tissue juice.						10 per cent tissue juice.						15 per cent tissue juice.					
Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.
19403	4.5	5.4	1.17	9	13	19418	2.85	4.59	1.6	7	15	19437	2.0	5.32	2.66	7	13	19470	2.5	8.92	3.57	13	18
19404	4.47	6.48	1.44	10	14	19419	3.71	9.46	2.58	7	16	19438	2.0	5.61	2.8	7	19	19471	2.30	9.66	4.2	11	25
19405	4.7	8.13	1.73	9	13	20043	2.47	5.93	2.41	10	16	20060	2.87	6.81	2.15	11	22	20084	3.02	8.55	2.84	11	27
20040	2.66	3.83	1.39	10	12	20044	2.21	4.20	1.9	10	18	20061	2.58	5.53	2.14	13	23	20085	4.78	11.21	2.34	12	28
20041	3.27	4.18	1.27	10	13	20045	2.9	5.71	1.97	10	16	20062	2.58	6.21	2.42	12	22	20086	3.05	10.02	3.28	10	24
20042	4.61	5.27	1.14	9	13																		
Average.....			1.36	9.5	13	Average.....			2.09	8.8	16.2	Average.....			2.43	10	19.8	Average.....			3.25	11.4	24.4

20 per cent tissue juice.						25 per cent tissue juice.						30 per cent tissue juice.						40 per cent tissue juice.					
Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$ Ratio.	Width of ring.	
	Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.		Control.	Experiment.
19484	2.34	5.89	2.53	9	17	19505	2.6	9.6	3.7	10	28	19511	3.66	15.0	4.07	10	31	19528	2.3	9.2	4.0	10	30
19485	3.59	8.52	2.47	12	20	19506	2.83	11.34	4.0	12	27	19512	3.94	15.0	3.8	10	32	19529	2.64	10.3	3.9	9	30
19509	4.2	16.09	3.81	11	33	19507	3.39	13.3	3.9	11	31	20001	2.33	9.08	3.9	10	33	19530	2.23	9.6	4.3	10	30
19510	4.18	15.68	3.80	11	35	20087	2.63	8.93	3.4	12	35	20002	3.5	11.79	4.17	9	32	19883	2.74	11.67	4.26	9	35
19998	3.0	10.97	3.65	12	31	20088	2.56	9.18	3.57	10	33	20003	2.9	10.0	3.45	14	30	19884	3.54	12.71	3.6	10	34
19999	3.17	9.52	3.02	11	27																		
20000	2.43	7.08	2.9	11	30	Average.....			3.7	11	30.8	Average.....			3.88	10.6	31.6	Average.....			4.01	9.6	31.8
Average.....			3.17	11	27.6																		

50 per cent tissue juice.						60 per cent tissue juice.						70 per cent tissue juice.						80 per cent tissue juice.					
Experiment No.	Relative increase.		$\frac{E}{C}$.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$.	Width of ring.		Experiment No.	Relative increase.		$\frac{E}{C}$.	Width of ring.	
	Control.	Experiment.	Ratio,	Control.	Experiment.		Control.	Experiment.	Ratio,	Control.	Experiment.		Control.	Experiment.	Ratio,	Control.	Experiment.		Control.	Experiment.	Ratio,	Control.	Experiment.
19531	2.36	9.20	3.9	10	26	19544	2.52	11.6	4.6	12	33	19546	2.47	11.36	4.6	9	33	19620	2.33	10.62	4.58	13	33
19533	2.74	13.2	4.8	10	31	19545	2.85	13.3	4.66	7	34	19547	2.82	13.4	4.75	10	33	19621	1.93	9.08	4.7	12	33
19886	2.17	9.77	4.5	14	36																		
19885	2.49	9.95	4.0	13	34	Average.....			4.6	9.5	33.5	Average.....			4.65	9.5	33	Average.....			4.64	12.5	33
20150	2.76	12.91	4.67	10	35																		
20151	2.10	8.74	4.16	11	34																		
Average.....			4.37	11.2	32.7																		

the concentration of the juice in the medium had reached 40 per cent, no further increase was observed when larger amounts were added. The results were checked by measurement of the width of the ring of the new tissue; that is, by comparing the absolute instead of the relative increases (Table VI). The arithmetic mean of the width of the ring of new tissue of several experiments, made with each dilution of juice, was plotted in ordinates, and the concentration of the juice in abscissæ (Text-fig. 7). The curve (Text-fig. 6) showed the same rapid increase of the rate of growth when a small amount of juice was added to the medium. There was no doubt that the substances used by the fibroblasts came from the embryonic juice and that the activity of the fibroblasts was a function of the concentration of the embryonic juice in the medium.

III.

SUMMARY.

The results of the investigation of the cause of the multiplication of fibroblasts *in vitro* may be summarized as follows: Although the life of fibroblasts in the plasma of an adult chicken which has fasted for 24 hours is not permanent, their proliferation is very active for some time. Are the substances used by the cells in their multiplication supplied by the plasma or by the tissue itself? In media composed of a constant amount of fibrin and of a mixture of Tyrode solution and serum in varied concentrations, the amount of growth appeared to be independent of the concentration, and even of the presence of serum. Serum was evidently not used by the cells. It was also found that fibrin is not utilized. This fact explains the results of the experiments of Lewis, who showed long ago that embryonic tissue can grow extensively in Locke solution. Then, the material from which the new cells are built must come from the tissue itself, as was previously supposed by Lewis, Ingebrigtsen, and Burrows. A comparative study of the growth in adult plasma of embryonic heart and of a 9 year old strain of fibroblasts led to the hypothesis that traces of embryonic juice stored in the original fragments are responsible for the activity manifested by the tissues during their temporary life.

When embryonic juice is added to the plasma of an adult chicken, the rate of multiplication of the fibroblasts increases and their life *in vitro* becomes permanent. Does the presence of embryonic juice determine the use by the cells of substances contained in adult plasma? The tissues were cultivated in media containing a constant amount of fibrin and embryonic juice, and varied concentrations of serum. The rate of growth was found to be independent of the amount of serum contained in the medium. It was also observed that the rate of growth in fibrin fixed in formaldehyde solution did not differ from that in normal fibrin. This fact demonstrated that embryonic juice does not give to the cells the power of using the constituents of plasma. When fragments of the 9 year old strain of fibroblasts were cultivated in media containing a constant amount of serum and fibrin, and varied concentrations of embryo juice, the rate of growth was found to be a function of the concentration of the embryonic juice in the medium. It was, therefore, evident that the material employed by the fibroblasts in their indefinite multiplication *in vitro* was supplied by the embryonic juice.

IV.

CONCLUSIONS.

1. It may be concluded that, under the conditions of the experiments and within the limits of accuracy of the method, the temporary multiplication of fibroblasts cultivated in the plasma of an adult animal is not due to the serum. It may be attributed to the presence of a small amount of embryonic juice within the tissue itself.

2. The indefinite multiplication of fibroblasts in a medium composed of adult plasma and of embryonic juice is due neither to the serum nor to the fibrin. It depends entirely on substances contained in the embryonic juice.

3. There is a definite relation between the rate of growth and the concentration of the embryonic juice in the medium.

CICATRIZATION OF WOUNDS.

XI. Latent Period.

By ALEXIS CARREL, M.D., and P. LECOMTE du NOÜY, Sc. D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The latent or quiescent period of cicatrization[1] extends from the time of traumatism to the beginning of contraction. During this stage of apparent inactivity, the mechanism which will bring about the redintegration of the tissues is progressively set in motion. A study of the latent period may lead to a better understanding of the secondary causes which directly or indirectly derive from the injury acting as primary cause, and are instrumental in starting regeneration. The purpose of this article is to examine the duration of the latent period, its transition to the period of contraction, and the characteristics of the curve expressing it.

I.

Method.

The experiments were made with wounds of geometric shape, obtained by excision of a flap of skin in the dorsal region of dogs. The animals were of medium size, short haired, and of quiet temper. 24 hours previous to the operation, they were given a warm bath after the hair had been clipped. Later on the same day, the skin of the dorsal region was washed a second time with soap and water. After the animal had been etherized, the skin was shaved, carefully washed with soap and warm water, and covered for 10 minutes with compresses soaked in 75 per cent alcohol. Then the animal was placed on the operating table and the skin was painted with tincture of 10 per cent iodine, which was allowed to dry for 10 minutes.

[1] Carrel, A., *J. Am. Med. Assn.*, 1910, lv, 2148.

The wounds were generally obtained by resection of a rectangular flap of skin. In some cases, circular flaps were extirpated by means of a sharp edged tube, 2 cc. in diameter. Hemostasis was secured by compression with gauze pads, or by temporary clamping of the small vessels. No ligatures were used, because after a few days they become centers of colonization for the bacteria existing on the surrounding skin. The measurement of the wounds was made in two different ways. In the earlier experiments, the width of a rectangular wound was ascertained with a compass and its variations were studied, instead of the change in the total area. In the more recent experiments, the area of the wound was measured, according to a technique previously described,[2] and expressed in square centimeters. An accurate appreciation of the area required a great deal of care. It is well known that the skin of the dog is not adherent to the aponeurosis, and is very mobile. This causes the size of the wound to become modified by slight changes in the position of the animal. Therefore, in spite of the precautions which were taken, variations occurred in the area of the wounds which were due only to experimental errors. At the end of the latent period, when the edges of the wound had become fixed to the bottom by granulation tissue, the method was much more accurate.

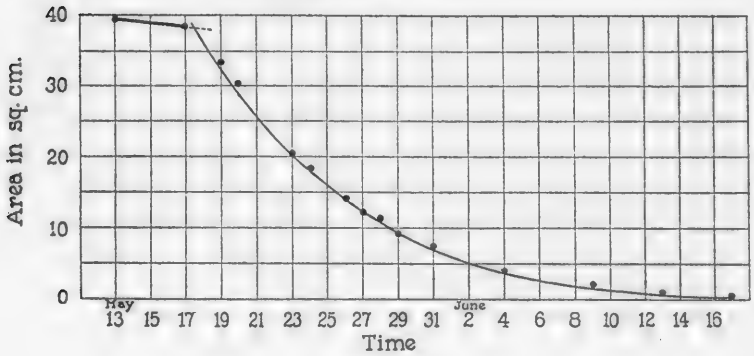
As the purpose of the experiments was to study the latent period under ordinary conditions, the dressing consisted only of talcum powder, paraffin, or plain gauze. The bacterial condition of the surface was ascertained merely by examination of films. Often the wound could be maintained in a condition of surgical asepsis, that is of mild infection, when the dressing was properly fixed to the skin. Infection was usually carried from the surrounding skin to the wound by the gauze, which moves about on the surface of the skin due to the constant movements of the animal. This could be prevented by stitching a gauze pad to the skin itself. Then a thick cotton pad was applied, fixed by bandages, and protected by a shirt.

Seven experiments are briefly described, and the appearance of the latent period is shown by Text-figs. 1 to 7. The width of the wound in centimeters, or its area in square centimeters, is plotted in ordinates and the time in abscissæ. The latent period is designated

[2] Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 430.

by the heavy line. The light line expresses the area of the wounds calculated by the formula of du Noüy,[3] which, of course, does not apply to the widths. In Text-figs. 5 to 7 the curves are arbitrary. The observed area, or width, is represented by the heavy points. In the first experiment, the latent period and the two subsequent periods are described, and the curve expresses the complete phenomenon from the excision of the flap to the last stage of epidermization. In the six other experiments, the latent period and the beginning of the contraction period only are shown.

EXPERIMENTS.



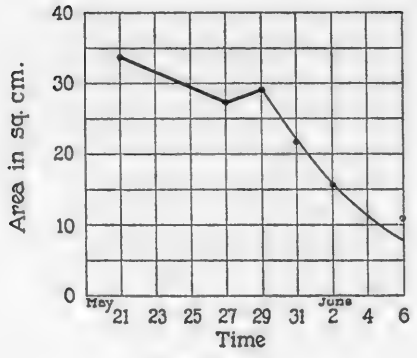
TEXT-FIG. 1.

Experiment 1. Latent Period and Periods of Contraction and Epidermization.— Very old female dog, about 13 years of age, No. 1. May 13, 1919. Resection of a rectangular flap of skin on dorsal region. Dressing with dry gauze. Subsequent dressings, dry gauze after washing with neutral sodium oleate. Dakin's solution compress when bacteria appeared in the films.

Date.....	May 13	17	19	20	23	24	26	27	28	29	31	June 4	9	13	17
Observed area....	39.4	38.7	33.5	30.2	20.3	18.7	14.3	12.2	11.5	9.2	7.7	4.0	2.7	1.1	0.5
Calculated area...					20.0	18.3	14.2	12.2	11.0	9.2	7.1	3.8	1.6	0.8	0.3

For the calculated curve, the index i was equal to 0.058.

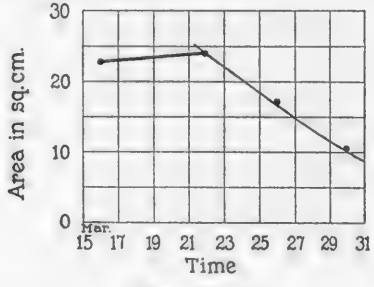
[3] du Noüy, P. L., *J. Exp. Med.*, 1916, xxiv, 451.



TEXT-FIG. 2.

Experiment 2. Latent Period and Abrupt Beginning of Contraction.—Female dog, about 2 years old, No. 2. May 21, 1919. Excision of a rectangular flap of skin. Dry gauze dressing. Washing with sodium oleate in subsequent dressings.

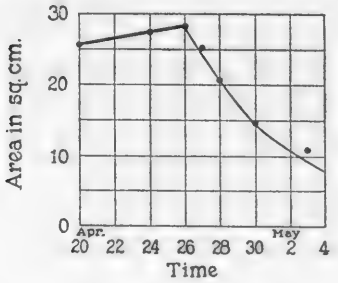
Date	May 21	27	29	31	June 2	6
Observed area	33.7	27.4	29.0	21.7	15.8	11.0



TEXT-FIG. 3.

Experiment 3. Latent Period and Period of Contraction.—Male dog, about 2 years old, No. 3. March 16, 1920. Excision of a rectangular flap of skin. Dry gauze dressings.

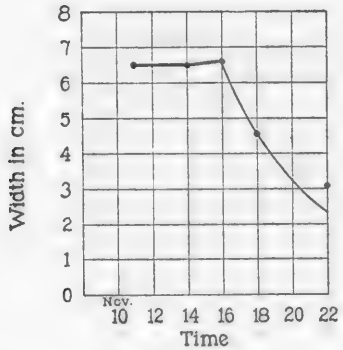
Date	Mar. 16	22	26	30
Observed area	23.0	24.0	17.3	10.6



TEXT-FIG. 4.

Experiment 4. Latent Period and Period of Contraction.—Male dog, about 1 year old, No. 4. April 20, 1920. Excision of flap of skin. Dry gauze dressing. Washing with neutral sodium oleate in subsequent dressings.

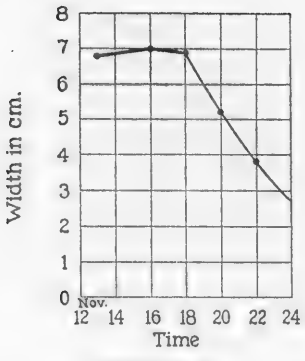
Date	Apr. 20	24	26	27	28	30	May 3
Observed area	25.8	27.4	28.4	25.1	20.7	14.7	11.0



TEXT-FIG. 5.

Experiment 5. Latent Period and Abrupt Beginning of Contraction.—Young male dog, about 11 months old, No. 5. November 11, 1907. Resection of a rectangular flap of skin in dorsal region. Measurement of the width of the wound with a compass. Talcum powder dressing. November 16. Beginning of granulations. Subsequent dressings, dry gauze.

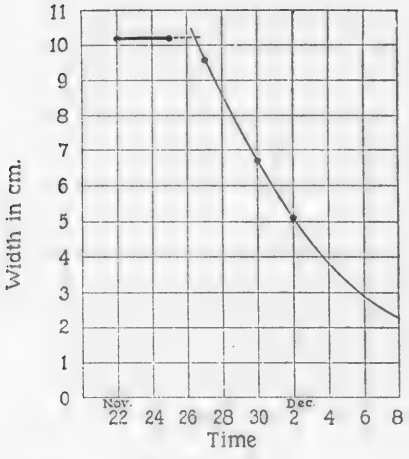
Date	Nov. 11	14	16	18	22
Width of wound	11.0	6.5	6.6	4.6	3.1



TEXT-FIG. 6.

Experiment 6. Latent Period and Abrupt Beginning of Contraction.—Young male dog, about 13 months old, No. 6. November 13, 1907. Resection of a rectangular flap of skin. Measurement of the width of the wound with a compass. Talcum powder dressing. November 18. Small irregular granulations.

Date	Nov. 13	16	18	20	22
Width of wound	6.8	7.0	6.9	5.2	3.8



TEXT-FIG. 7.

Experiment 7. Latent Period and Period of Contraction.—Young male dog, about 10 months old, No. 7. November 22, 1907. Resection of a rectangular flap of skin. Measurement of wound with a compass. Talcum powder dressing. November 27. Granulations began.

Date	Nov. 22	25	27	30	Dec. 2
Width of wound	11.0	10.2	9.6	6.7	5.1

II.

DISCUSSION.

The duration of the latent period varied from 5 to 7 days. It was found, in the course of many other experiments, that the limits of variations may be wider. While the subsequent stages of cicatrization are not affected by external factors, it appears that the latent period may be easily modified in its duration by many local causes, such as mechanical irritation of the tissues, infection, and even by the diet of the animal, as was shown by Clark.[4] The experiments described above deal only with the latent period under ordinary conditions of mild bacterial and mechanical irritation, which was generally obtained by dry gauze dressings. During the first stage of healing, the area was generally found to remain constant. However, in Experiment 7 an increase of the surface, and in Experiment 5 a decrease followed by an increase of the surface, were observed. These changes were probably due to errors of observation. As long as the edges of the wound were not fixed to the bottom by granulating tissue, a slight modification of the tension of the surrounding skin could modify the area considerably. The appearance of the granulation tissue generally announced the end of the latent period. But there was no definite relation between both phenomena. The end of the latent period was marked not only by the growth of granulation tissue, but by an abrupt beginning of the contraction period, which immediately acquired its maximum velocity. In Experiments 4 and 7, there was a transition period which lasted possibly 24 hours, and during which the contraction started slowly. As a rule, the onset of contraction was sudden and a period of maximum activity succeeded the period of complete quiescence.

The formula of du Noüy was found to apply accurately to the beginning of the period of contraction. On the graphs, it may be seen that the light line representing the calculated area follows the observed area almost constantly. There was a perfect coincidence between the calculated and the observed surfaces in Experiment 1, even during the first hours of the contraction. An examination of the seven graphs shows that the phenomenon could not be accurately

[4] Clark, A. H., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 117.

expressed by a curve presenting a point of inflexion. So far, this fact has not been emphasized, and, on account of an insufficient description of the conditions of the experiments, certain curves, in previous publications, were misleading.[5] Recently, Fauré-Frémiet and Vlès[6] attempted to apply to the cicatrization process the equation expressing the monomolecular autocatalytic reaction used by Robertson[7] in his studies on growth. As a consequence of this hypothesis, the curve of the areas must pass by a point of inflexion, in conformity with the equation

$$\frac{S}{S_o - S} = e^{KSo\,(T - t_{\frac{1}{2}})}$$

which necessitates that the rate should be maximum at the time $t_{\frac{1}{2}}$ where $S = \frac{S_o}{2}$. One of the curves calculated in this manner agrees fairly closely with that of du Noüy. The other presents a point of inflexion; that is, a maximum in the rate of healing during the period of contraction. There is evidently a close relation between this curve and the observed curve of an experiment previously reported.[5] But the point of inflexion of this observed curve was due to a mere accident. The wound was old and infected, and had begun to cicatrize under a plaster of Paris splint. The observed curve showed the variations of the rate of cicatrization both before and after normal cicatrization had really begun. As these details were not mentioned in the paper, Fauré-Frémiet and Vlès[6] could not help being misled by this accidental fact. Although their formula and the formulas published by de Beaujeu[8] and Lumière[9] show a lack of agreement with the observed facts, there is no doubt that the curve representing the normal process of cicatrization may be expressed by more than an equation, as has already been pointed out by du Noüy.[10]

[5] Carrel, A., and Hartmann, A., *J. Exp. Med.*, 1916, xxiv, 432.

[6] Fauré-Frémiet, E., and Vlès, F., *Compt. rend. Acad.*, clxviii, 363.

[7] Robertson, T. B., Principles of biochemistry, Philadelphia, 1920, 475.

[8] de Beaujeu, J., *J. Exp. Med.*, 1917, xxvi, 81.

[9] Lumière, A., *Bull. Acad. méd.*, 1918, lxxix, series 3, 213; *Rev. chir.*, 1917, liii, 656.

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But these equations must apply as well to the beginning of the contraction period as to the end of the epidermization period. A glance at the figures and at the chart expressing the observed and calculated areas of Experiment 1 shows that the coincidence is almost perfect and quite satisfactory, in view of the percentage error necessarily involved in experimental wounds on dogs.

Several equations have already been applied by du Noüy to the curve of cicatrization. The first[11] was expressed as follows:

$$T = K_1 \operatorname{Log}_e \frac{S_o}{S} + 2K_2 \left(\sqrt{S_o} - \sqrt{S}\right)$$

The second[12] has proven more satisfactory:

$$S = S_o\, e^{-K\left(T + \frac{T^2}{2p}\right)}$$

The figures calculated by this last equation coincide almost exactly with those calculated by means of the well known extrapolation formula:

$$S_n = S_{n-1}\left[1 - i\left(t + \sqrt{t + nt}\right)\right]$$

They applied to the first stage of contraction as accurately as to the later periods of cicatrization.

III.

CONCLUSIONS.

1. The latent period of cicatrization varies generally from 5 to 7 days.

2. It stops abruptly and contraction starts with its maximum velocity.

3. The formula of du Noüy applies to the beginning of the contraction period as well as to the subsequent periods.

[11] du Noüy, P. L., *J. Exp. Med.*, 1917, xxv, 722.

[12] du Noüy, P. L., *J. Exp. Med.*, 1919, xxix, 329, Text-fig. 4.

THE DIASTATIC ACTIVITY OF THE BLOOD IN EXPERIMENTAL HYPERGLYCEMIA.

By HOWARD T. KARSNER, M.D., HERBERT L. KOECKERT, M.D., AND SPENCER A. WAHL, M.D.

(*From the Department of Pathology of the School of Medicine, Western Reserve University, Cleveland.*)

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I.

INTRODUCTION.

Since the demonstration of diastatic activity of the blood and lymph by Magendie, Bial, and Röhman, the production of hyperglycemia and glycosuria by Claude Bernard, and the demonstration of the influence of the pancreas upon carbohydrate metabolism by von Mering and Minkowski, numerous investigators have conducted further studies. It has long been known that the injection of such substances as adrenalin, strychnine, morphine, and more recently, saliva, pituitrin, and various other glandular extracts possessing amylolytic properties leads in some instances to the development of hyperglycemia and glycosuria. In spite of considerable conflict concerning the diastatic content of the blood in these conditions the mass of evidence so far accumulated supports the belief that little or no change is produced. However, Watanabe found in rabbits that the intravenous injection of human saliva and the intraperitoneal administration of sodium bicarbonate (5 or 10 gm.) each produced an increase, while the parenteral administration of soluble starch and the intravenous injection of sodium bicarbonate (5 or 10 gm.) each produced a slight increase in the diastatic activity of the blood.

Further observations have been made concerning the rôle of the pancreas. Following ligation of the duct of Wirsung, Schlesinger reported an increase in the diastatic activity of the blood; Clerc and Loeper verified this result in rabbits; Gould and Carlson, working with dogs, ligated both pancreatic ducts and found a marked increase in the diastatic power of the serum, which they assumed to be due to absorbed amylopsin; King observed an increased diastatic activity in the serum and urine upon ligation of both pancreatic ducts. Wohlgemuth, Noguchi, and King reported a quantitative increase in the diastatic activity of the urine following ligation of both pancreatic ducts.

Bainbridge and Beddard, as well as Carlson and Luckhardt, reported that in cats the concentration of blood diastase is not materially changed by excision of the pancreas; Schlesinger states that pancreatectomy leads to complete disappearance of amylase from the blood. Otten and Galloway found that in dogs immediately following pancreatectomy the concentration of blood diastase sinks rapidly, then rises slightly, remaining at a constant level for a time but never returning to the normal level again. King reports a reduction in the amount of blood diastase in depancreatized dogs. Milne and Peters obtained a decided increase in the blood diastase after complete pancreatectomy.

Recently, the question of the diastatic activity of the blood has been more accurately studied in human diabetes. Myers and Killian (1917) reported an increase in the diastatic activity of the blood in cases of human diabetes. Still more recently (November, 1920), Lewis and Mason, employing essentially the same method as Myers and Killian, found a distinct decrease in the blood diastase in human diabetes.

II.

Method.

The methods for the determination of the diastatic activity of the blood have varied from time to time but all depend upon a hydrolytic process. Wohlgemuth (1908) based his method upon the hydrolysis of starch into erythrodextrin by amylase, using the simple iodine reaction. Myers and Killian (1917) devised a method based upon the hydrolysis of starch into glucose, using Myers and Bailey's modification of the Lewis-Benedict method for the blood sugar determination.

In our laboratory the method used was essentially the same as that described by Myers and Killian with the exception of the substitution of Folin and Wu's method for blood sugar determination.

Our experiments consisted (1) in observing the effect upon blood diastase in rabbits after the subcutaneous injection of phlorhizin, uranyl nitrate, morphine sulfate, and adrenalin; (2) in noting the effect of puncture of the floor of the fourth ventricle; and (3) in observing the effect upon the content of blood diastase in completely depancreatized dogs. The controls consisted in noting (1) the effect of diet; (2) the effect produced by repeated bleeding of the animal comparable to the procedure followed in the experiment; (3) the effect of asphyxia; (4) the effect of ether anesthesia; and (5) the effect of a surgical operation similar in extent to pancreatectomy.[1]

[1] All operations were performed under ether anesthesia.

The rabbits were fed upon a diet consisting of carrots, oats, and water; the diet for the dogs was composed of a balanced mixture of beef heart, casein, crackers, salt, lard, and water. The specimens of blood were taken from the ear veins of the rabbits and by cardiac puncture from the dogs. All blood diastase determinations were started within 10 minutes after withdrawal of the blood. The examinations in the case of the rabbits were made 2 hours following the injection and the puncture; and then at intervals of 24 hours until the blood sugar had returned to normal or the animal died. After pancreatectomy in dogs the examinations were made immediately following the operation in two cases; in all animals 2 hours later, and then at 12 hour intervals until the animal succumbed.

All drugs were given subcutaneously in the following doses: morphine sulfate, 2 cc. of a 4 per cent solution (80 mg.); adrenalin chloride, 2 cc. of 1: 1,000 solution; uranyl nitrate, 3 mg.; phlorhizin, 0.25 gm. The Eckhard operation was performed in two of the successful puncture experiments. The pancreatectomies consisted in complete removal of the pancreas by usual operative technique under ether anesthesia without morphine.

III.

EXPERIMENTS.

Three rabbits were given phlorhizin subcutaneously in doses of 0.25 gm. dissolved in warm distilled water. In one animal with a normal diastatic activity ranging between 3 and 8 mg. per 100 cc. the first injection of phlorhizin produced no change in diastatic activity, but a second injection 5 days later was followed in 24 hours by a figure for diastase of 20 mg. At this time, however, that animal had been bled ten times in 28 days and the result was probably in part influenced by the repeated bleedings, and therefore can only be regarded as indicating a tendency to increase. In another animal with normal figures of 2 to 5 mg. the first injection of phlorhizin was followed by a rise to 11 mg., again a tendency to increase, but a second injection in the same animal showed no change. A third animal showed no change following two separate injections. Table I is the protocol of one animal.

TABLE I

Effect of the Subcutaneous Injection of Phlorhizin. Rabbit 1.
Weight 1.2 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Diastase* per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	*a.m.*	*mg.*	*mg.*			
July 22–Aug. 7..		112 139	3 8	Negative.	Negative.	Normal amounts with minimal and maximal of six determinations.
Aug. 12.....	11.30 *p.m.*					Phlorhizin, 0.25 gm. subcutaneously.
	1.30	122	4			
" 13.....	1.30	131	9	Negative.	Negative.	
" 14.....	1.30 *a.m.*	128	3	Positive.	"	
" 20.....	11.30 *p.m.*					Phlorhizin, 0.25 gm. subcutaneously.
	1.30	137	20	Positive.	Negative.	High diastase probably due to repeated bleedings.
" 21.....	1.30	128	9	"	"	
" 22.....	1.30 *a.m.*	137	14	"	"	
" 30.....	11.30 *p.m.*					Phlorhizin, 0.25 gm. subcutaneously.
	1.30	122	9	Positive.	Positive.	
" 31.....	1.30	115	2	"	"	

* Throughout the tables "diastase" refers to the amount of sugar produced by the action of the blood upon soluble starch.

Four rabbits were given uranyl nitrate in doses of 0.003 gm. of pure salt. All four showed albuminuria and glycosuria, and all were fatally intoxicated and showed acute nephritis at autopsy. None of these showed hyperglycemia and none showed any increase in diastatic activity of the blood even when moribund. Table II is the protocol of one animal.

Five rabbits were given doses of 0.080 gm. of morphine sulfate subcutaneously. Collectively fourteen injections of morphine were given. All showed some degree of hyperglycemia, three showed

glycosuria, and all showed respiratory disturbance. A definite increase in diastase occurred when severe hyperglycemia was produced. Table III is a protocol of one animal.

Six rabbits were given adrenalin (Parke, Davis and Company) in doses of 2 cc. of 1:1,000 solution from a freshly opened bottle. Collectively eight injections were given. All showed hyperglycemia

TABLE II.

Effect of the Subcutaneous Injection of Uranyl Nitrate. Rabbit 2.
Weight 1.1 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	*a.m.*	*mg.*	*mg.*			
Aug. 25–Sept. 3.		110 192	4 10	Negative.	Negative.	Normal amounts with maximal and minimal of six determinations.
Sept. 17.....	11.00					Uranyl nitrate, 3 mg. subcutaneously.
	p.m.					
	1.00	128	4	Negative.	Negative.	
" 18.....	1.00	125	4	"	"	
" 19.....	1.00	156	4	"	"	
" 20.....	1.00	172	6	Positive.	"	
" 21.....				"	"	
" 22.....	1.00	185	8	"	0.3 per cent.	
" 23.....				"	Negative.	
" 24.....	1.00	140	7	"	"	
" 25.....				"	"	
" 26.....						Died. Acute tubular nephritis.

and glycosuria. In one animal with control figures ranging between 4 and 6 mg. per 100 cc. the first injection of adrenalin was followed by a figure of 11 mg. for blood diastatic activity. All others showed no increase. Table IV is a protocol of one animal.

Puncture of the floor of the fourth ventricle was performed in three animals, one by the Claude Bernard method and two by the Eckhard method. All showed hyperglycemia, two showed gly-

cosuria, and none showed any definite increase in diastatic activity. Table V is the protocol of one animal.

Seven rabbits were placed on diets alternately of green vegetables and of carrots, oats, and water. There was no alteration

TABLE III.

Effect of the Subcutaneous Injection of Morphine Sulfate. Rabbit 3.
Weight 1.6 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	*a.m.*	*mg.*	*mg.*			
July 20–Aug. 7..		125 180	3 13	Negative.	Negative.	Normal amounts with maximal and minimal of six determinations.
Aug. 11.....	8.30					Morphine sulfate, 2 cc. of 4 per cent solution (80 mg.) subcutaneously.
	10.30	320	16			
" 12.....	10.30	135	12	Negative.	Negative.	
" 13.....	10.30	125	9	"	"	
" 19.....	8.30					Morphine sulfate, 2 cc. of 4 per cent solution (80 mg.) subcutaneously.
	10.30	419	35	Negative.	1.5 per cent.	
" 20.....	10.30	132	12			
" 21.....	10.30	139	6			
" 30.....	8.30					Morphine sulfate, 2 cc. of 4 per cent solution (80 mg.) subcutaneously.
	10.30	402	41			
" 31.....	10.30	114	10	Negative.	1.8 per cent.	
Sept. 1.....	10.30	138	9			

of blood sugar or of diastatic activity of the blood. Table VI is the protocol of one animal.

Two rabbits were bled repeatedly. The hemoglobin was markedly reduced. Unfortunately the cell counts were not determined at the beginning of the experiment but the figures given indicate a marked

reduction. Associated with the anemia was an increase in blood sugar, as has been found by Hirsch. There was also an increase in diastase as indicated in Table VII.

TABLE IV.

Effect of the Subcutaneous Injection of Adrenalin Chloride. Rabbit 4.
Weight 1.8 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	*a.m.*	*mg.*	*mg.*			
July 26–Aug. 7..		123 160	3 6	Negative.	Negative.	Normal amounts with maximal and minimal of six determinations.
Aug. 11.....	8.30					Adrenalin chloride, 2 cc. of 1: 1,000 solution subcutaneously.
	10.30	446	5			
" 12.....	10.30	124	7	Negative.	2.0 per cent.	
" 13.....	10.30	232	7	"	Negative.	
" 19.....	8.30					Adrenalin chloride, 2 cc. of 1: 1,000 solution subcutaneously.
	10.30	579	11			
" 20.....	10.30	162	6	Negative.	1.8 per cent.	
" 21.....	10.30	192	6			
" 30.....	8.30					Adrenalin chloride, 2 cc. of 1: 1,000 solution subcutaneously.
	10.30	427	9			
" 31.....	10.30	138	2	Negative.	2.5 per cent.	
Sept. 1.....	10.30	129	4	"	1.7 " "	
" 2.....	10.30	121	4	"	Negative.	

Nine rabbits were definitely asphyxiated either by smothering or by placing them in the Benedict animal calorimeter under low oxygen tension. In one no hyperglycemia was produced; in six moderate hyperglycemia resulted with a tendency to increase in the amount of blood diastase in two. In two marked hyperglycemia with

TABLE V.

Effect of Puncture of the Floor of the Fourth Ventricle. Rabbit 5.

Weight 1 kilo.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	*a.m.*	*mg.*	*mg.*			
Sept. 14–24.....		120 129	7 9	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
Oct. 4.....	11.45					Bernard puncture of floor of fourth ventricle.
	p.m.					
	1.45	536	8			
" 5.....	1.45	90	10	Negative.	0.5 per cent.	
" 6.....	1.45	110	6	"	Negative.	
" 10.....						Died.

TABLE VI.

Effect of Diet. Rabbit 6.

Weight 1 kilo.

Date.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
			Albumin.	Sugar.	
1920	*mg.*	*mg.*			
July 21.....	155	8	Negative.	Negative.	After green vegetable diet for 5 days.
" 23.....	140	5			After green vegetable diet for 7 days.
" 26.....	168	4			After carrots, oats, and water for 3 days.
" 30.....	137	5			After carrots, oats, and water for 7 days.

a definite increase in the concentration of blood diastase was produced. Table VIII is the protocol of one rabbit.

Total excision of the pancreas was successfully performed in three dogs, one of which lived 36 hours, another about 48 hours, and a third about 56 hours. All showed hyperglycemia, glycosuria, and marked

TABLE VII.

Effect of Repeated Bleeding. Rabbit 7.

Weight 0.75 kilo.

Date.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
			Albumin.	Sugar.	
1920	*mg.*	*mg.*			
Oct. 15–17....	91 114	2 4	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
" 26.....	140	4			After 9 days intermission.
" 27.....	125	7			
" 28.....	100	3			
Nov. 4.....	97	4			After 7 days intermission.
" 5.....	119	3			
" 6.....	115	3			
" 12.....	123	3			After 6 days intermission.
" 13.....	117	7			
" 14.....	121	4			Red blood cells 6,000,000; hemoglobin (Sahli) 55 per cent.
" 18.....	148	15			After 4 days intermission.
" 19.....	144	16			
" 20.....	158	15			
" 24.....	205	10			After 4 days intermission.
" 25.....	205	15			Red blood cells 5,360,000; hemoglobin (Sahli) 55 per cent.
" 26.....	163	13			

TABLE VIII.

Effect of Asphyxia. Rabbit 8.

Weight 1.2 kilos.

Date.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
			Albumin.	Sugar.	
1921	*mg.*	*mg.*			
Jan. 20.....	122	4	Negative.	Negative.	Normal.
" 21.....	107	7			"
" 22.....	81	4			"
Feb. 7.....	347	19			Asphyxiated in Benedict tank; low oxygen tension for ½ hr.
" 8.....			Negative.	1.4 per cent.	

increase in diastatic activity of the blood. The dog illustrated in the protocol (Table IX) was free from general infection, one other showed a slight general fibrinous peritonitis, and one showed gangrene of the duodenum. The results, however, were parallel in all three animals.

TABLE IX.

Effect of Pancreatectomy. Dog 1.

Weight 11 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	*a.m.*	*mg.*	*mg.*			
Nov. 18–22.....		168 179	13 17	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
" 23.....						Complete pancreatectomy; finished 11 a.m.
	11.00	319	17			Blood at close of operation.
	p.m.					
	1.00	241	16			Blood 2 hrs. after operation.
	9.00	365	23	Negative.	2.3 per cent.	Blood 10 hrs. after operation.
	a.m.					
" 24.....	11.00	500	26	"	4.0 " "	Blood 24 hrs. after operation.
" 25.....	11.00	810*	45	"	5.0 " "	Blood 48 hrs. after operation.
" 26.....						Animal found dead in cage.

*This high figure was determined by identically the same method as the others. The other two animals showed maximum figures for blood sugar of 416 and 540 mg., and maxima for diastatic activity of 53 and 31 mg.

Two dogs were completely anesthetized with ether for 1 hour. A slight increase in blood sugar and in diastatic activity of the blood was found; both of these were of short duration. In one animal

with control figures for diastatic activity of 3 to 13 mg. per 100 cc., the ether anesthesia was followed 10 hours later by a figure of 22 mg. That this is not constant will be seen by reference to the protocol of the animal in which enteroenterostomy was performed (Table XI). The other experiment with ether is shown in Table X.

TABLE X.

Effect of Ether Anesthesia. Dog 2.

Weight 10 kilos.

Date.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
			Albumin.	Sugar.	
1920	*mg.*	*mg.*			
Nov. 7–9.....	105	4	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
	134	13			
" 16.....	163	14			Before anesthesia.
					Ether " 9.30–10.30 a.m. (1 hr.).
	240	12			Blood 2 hrs. following anesthesia.
	240	18	No urine.		Blood 10 hrs. following anesthesia.
" 17.....	153	8	Negative.	Negative.	Blood 24 hrs. following anesthesia.
" 18.....	143	5	"	"	Blood 36 hrs. following anesthesia.
	137	8			Blood 48 hrs. following anesthesia.

A dog was subjected to lateral enteroenterostomy. The operation required 1 hour and was followed by an uneventful recovery. Table XI shows that there was a tendency to hyperglycemia but no increase in diastatic activity of the blood.

TABLE XI.

Effect of Enteroenterostomy Operation. Dog 3.

Weight 12 kilos.

Date.	Hr. blood was drawn.	Blood sugar per 100 cc.	Diastase per 100 cc.	Urine.		Remarks.
				Albumin.	Sugar.	
1920	*a.m.*	*mg.*	*mg.*			
Dec. 14–16.....		89 109	8 12	Negative.	Negative.	Normal amounts with maximal and minimal of three determinations.
" 20.....						Enteroenterostomy (1 hr. duration).
	12.00	222	7			Blood immediately following operation.
	p.m. 2.00	161	5			Blood 2 hrs. following operation.
	12.00	115	13	Negative.	Negative.	Blood 12 hrs. following operation.
	a.m.					
" 21.....	12.00	119	5			Blood 24 hrs. following operation.
	p.m. 12.00	136	4	Negative.	Negative.	Blood 36 hrs. following operation.
	a.m.					
" 22.....	12.00	130	6			Blood 48 hrs. following operation.
" 23.....	12.00	114	12			Blood 72 hrs. following operation.

IV.

SUMMARY.

Upon the subcutaneous injection of phlorhizin and uranyl nitrate, their action being checked by the production of glycosuria, no constant change was produced in the concentration of the blood diastase. The adrenalin and puncture of the floor of the fourth ventricle both produced a marked transitory hyperglycemia and glycosuria without altering the content of blood diastase. On the other hand, morphine, when producing a marked definite hyper-

glycemia, caused also a decided increase in the concentration of the blood diastase.

By varying the diet of normal rabbits no material change was produced in the amount of blood diastase. By bleeding normal rabbits periodically as carried out in the experiments, there was no effect upon the diastatic activity until definite hemoglobinemia was produced; then there was a tendency to increase in the diastatic content simultaneously with the rise in blood sugar.

In order to determine the factors leading to the increase in blood diastase by the injection of morphine, the effect of asphyxia was noted. In the asphyxiated as well as in the morphinized animals when marked hyperglycemia was produced there resulted a decided increase in the concentration of the blood diastase. We have no ground for an opinion as to whether or not morphine hyperglycemia is due to asphyxia. All our morphinized animals showed respiratory depression and it appears at least possible that the increase in diastatic activity may have something in common with that of asphyxia.

Upon complete removal of the pancreas in dogs the hyperglycemia and glycosuria were progressive until death and were accompanied by a definite decided increase in the diastatic activity of the blood, the rise occurring at varying intervals following the operation in the different animals; but in no way was the increase in the diastase proportional to the amount of blood sugar.

Except for a slight tendency to increase 12 hours following the anesthesia, the ether anesthesia administered for 1 hour produced no definite change in the diastatic content of the blood, even though a slight hyperglycemia was immediately produced. The control operation performed consisted in an enteroenterostomy of 1 hour's duration, as a result of which the diastatic content was not changed from the normal. The slight hyperglycemia produced was undoubtedly the result of ether anesthesia.

Watanabe points out the great variation in the diastatic activity of the blood of rabbits, which is also found in our experiments. The same applies to the dog. In both animals the normal diastatic activity is greater than that of man.

If it prove true upon more extended study that human diabetes is associated with an increase in diastatic activity of the blood, as

is indicated by the studies of Myers and Killian and of DeNiord and Schreiner, it would seem that the experimental forms of this disease produced by pancreatectomy and perhaps also by asphyxia and morphine administration more nearly approximate the human disease than do other forms of experimental hyperglycemia. It is suggested that when inferences are drawn from experimental studies of animals these similarities and dissimilarities should be taken into consideration.

V.

CONCLUSIONS.

1. The diastatic activity of the blood is not materially changed by the subcutaneous injection of phlorhizin, uranyl nitrate, or adrenalin.

2. The diastatic activity of the blood is not affected by puncture of the floor of the fourth ventricle.

3. The diastatic activity of the blood is definitely increased by subcutaneous injections of morphia when a marked hyperglycemia is produced. The same phenomenon appears in asphyxia.

4. Complete pancreatectomy in dogs produces an increase in the diastatic activity of the blood.

5. The diastatic activity of the blood is not altered by the diet.

6. Ether anesthesia, alone, produces a very slight tendency to increase in the diastatic activity of the blood.

7. Repeated periodic small hemorrhages (4.5 cc.) produce no change in the diastatic activity of the blood until a definite color anemia results, when a slight tendency toward increase occurs simultaneously with the rise in blood sugar.

8. The diastatic activity of the blood is not proportional to the concentration of the sugar in the blood.

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TYPHUS FEVER AMONG RECENT IMMIGRANTS.

Experimental Demonstration of the Identity of This Disease with European Epidemic Typhus Fever.

By PETER K. OLITSKY, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plate 25.

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In January, 1921, through the kindness of Dr. S. B. Wolbach of Harvard Medical School, we received guinea pigs in which two strains of typhus virus were being propagated. One of these strains was originally derived from the blood taken from a patient during the height of typhus fever; the other, from the saline solution washings of a cage harboring typhus-infected lice. In July, 1920, with these materials, Dr. Wolbach, working in Warsaw, Poland, induced experimental typhus fever in guinea pigs; in the next half year he transmitted it through about fifteen successive animals and determined the existence of a cross-immunity between the human and louse strains.

In February, 1921, when we were engaged in an experimental study of the virus of typhus fever, employing for this purpose the guinea pigs given to us by Dr. Wolbach, a number of immigrants arrived at the Port of New York from different parts of Europe and showed a syndrome of headache, continued fever, petechial rash, positive Weil-Felix reaction[1,2] and infestation with body lice, which was suggestive of typhus fever. Through the kindness of Dr. John E. Holt-Harris, Director of Laboratories at the Quarantine Station, we were enabled

[1] Weil, E., and Felix, A., *Wein. klin. Woch.*, 1916, xxix, 974; 1917, xxx, 1511. This test is based on the observation of Wilson (Wilson, W. J., *J. Hyg.*, 1909, ix, 332; 1910, x, 155) that agglutinins for intestinal, colon-like bacilli are present in the serum of typhus fever patients.

[2] Dr. John E. Holt-Harris, Director of Laboratories at the Quarantine Station, applied the Weil-Felix test to these patients and found definite agglutination reactions in all—indeed, in some instances in dilutions of 1: 1,280.

to obtain blood from an immigrant suspected of having typhus fever. We inoculated the blood into guinea pigs in which was set up an experimental disease which we were thus able to compare with the experimental disease being propagated in these animals with the Polish virus.

Herewith is presented a detailed study of the experimental disease induced in the guinea pig by injecting the blood from this immigrant and the results of a comparison of this experimental disease with that set up in the same animal with the human and louse strains of the Polish virus.

Source of Material.

The patient from whom material was collected for the following experiments was a young, adult female, a native of Czecho-Slovakia, who had embarked for New York at Trieste, Italy. At the time of examination she had been ill for 8 days with headache and continued fever. Her face showed the suffusion and her conjunctivæ the injection characteristic of typhus fever, and the anterior surfaces of her forearms and arms were covered with a fading petechial rash. The Weil-Felix reaction, performed by Dr. Holt-Harris, was positive in a serum dilution of 1:320. Body lice infested the patient before admission to the hospital.

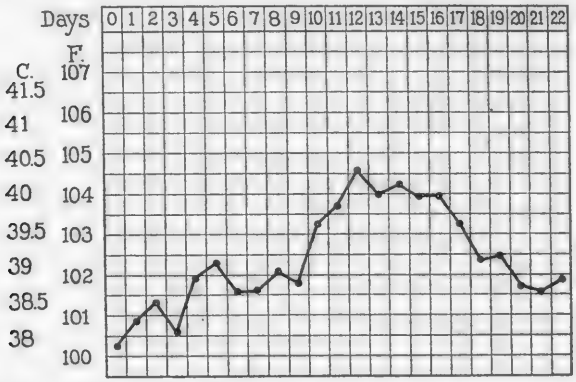
Transmission to Guinea Pigs.

Blood was obtained on the 8th day of illness, by venous puncture with a sterile syringe previously washed in 50 per cent sodium citrate solution, and 3 cc. of the blood, which proved sterile by aerobic cultivation tests, were immediately injected intraperitoneally into each of two guinea pigs, A and B.

Protocol 1.—Guinea Pig A, after 19 days during which the average temperature was 38.5°C., showed on the 20th day a rise of temperature to 40.4°C. It was then bled by heart puncture with a needle and syringe prepared in the usual manner and 3 cc. of the blood, sterile by aerobic tests, were injected into each of three normal guinea pigs. The rise of temperature in the second series occurred, on an average, 11 days after injection, when the blood, in the same amount, was transferred to a third group. In this way we have succeeded in transmitting the fever through at least nine successive animals, with an average incubation period of 9 days.

Guinea Pig B showed a rise in temperature to 40.4°C. on the 7th day after injection, when it was bled and the blood transferred to three normal animals. The guinea pigs of this and subsequent passages (second to fourth) yielded profuse growths of *B. proteus* from the heart's blood and this series was, therefore, discontinued and the results were discarded.

The febrile reaction in the guinea pigs which were allowed to recover lasted from 4 to 8 days. Thereafter the temperature promptly fell to normal and the animals remained well (Text-fig. 1).



TEXT-FIG. 1. Fifth guinea pig passage of the disease induced by the blood of the immigrant. Incubation period 9 days; fever 8 days.

The guinea pigs killed during the febrile reaction showed the following pathological picture.

Macroscopic Examination.—All the organs were normal in appearance with the exception of the spleen which was slightly enlarged, darker than normally, and of which the lymphoid follicles were prominent and raised above the surface. Occasionally the spleen was not enlarged. The skin, because of its hairiness, showed no definite lesions on its outer surface. The inner surface, however, especially after the corium had been stripped off, revealed occasional petechial spots.

Microscopic Examination.—The central nervous system showed characteristic lesions. There was no meningeal involvement except in the case of its blood vessels, which showed the changes described below. The gray matter of the brain, especially that of the midbrain, contained many small, localized hemorrhages. The striking changes were microscopic and consisted of focal accumulations of cells about the capillaries and arterioles. These lesions have been described in

detail by Ceelen,[3] Nicol,[4] and others in European typhus fever, by Doerr[5] and others in the experimental disease in guinea pigs, and recently by Wolbach and Todd[6] in Mexican typhus fever. The nodules consisted mainly of macrophages, or endothelial leucocytes of Mallory,[7] and a few leucocytes, mononuclears, and polymorphonuclears, and were always to be found in close proximity to the vessels. The vessels were surrounded by a more or less dense collar of the macrophages or endothelial leucocytes and leucocytes of the mononuclear and polynuclear type, which at some points was sufficient to form a distinct nodular mass giving rise to the *periarteriolitis nodosa* described by Nicol[4] (Fig. 1). The endothelium of the vessels, often markedly swollen, was necrotic in some portions and proliferated in others; at times the endothelial cells filled the lumen. In other arterioles and capillaries occluding thrombi were sometimes demonstrable.

The heart tissue showed the vascular lesions described by Nicol:[4] localized necrosis of the intima, swelling and proliferation of endothelial cells, thrombus formation, perivascular accumulations of endothelial leucocytes, polymorphonuclears, and mononuclears to form, in some areas, a *periarteriolitis nodosa*, focal nodules of these cells appearing in the interfibrillary spaces usually in proximity to the vessels. Besides, there were a number of small localized hemorrhages (Fig. 2).

The structure of the spleen obscured the vascular picture, but the organ showed, as a rule, congestion and leucocytic infiltration of the splenic pulp, as noted by Nicol.[4] The lymphoid follicles were enlarged and at the center a number of endothelial leucocytes were noted in active phagocytosis, a picture not unlike that seen by Mallory[8] in Peyer's patches in typhoid fever.

The skin showed in the corium nodular areas similar to those found in the brain and heart. These were near the capillaries and arterioles which showed the changes in the endothelium and the thrombus formation described above. In addition there were small, localized hemorrhages (Fig. 3). Similar vascular lesions in the skin of man have been described by Fraenkel,[9] Aschoff,[10] von Chiari,[11] and others in cases of typhus fever in Europe, by Wolbach and Todd[6] in those in Mexico, and by Löwy[12] in experimental typhus fever of guinea pigs.

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[3] Ceelen, *Berl. klin. Woch.*, 1916, liii, 530.

[4] Nicol, K., *Beitr. path. Anat. u. allg. Path.*, 1919, lxv, 120.

[5] Doerr, R., *Centr. Bakt., 1te Abt., Orig.*, 1921, lxxxv, Nos. 6 and 7, p. 2.

[6] Wolbach, S. B., and Todd, J. L., *Ann. Inst. Pasteur*, 1920, xxxiv, 153.

[7] Mallory, F. B., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 69.

[8] Mallory, F. B., *J. Exp. Med.*, 1898, iii, 611; The principles of pathologic histology, Philadelphia and London, 1920, 170.

[9] Fraenkel, E., *Münch. med. Woch.*, 1914, lxi, 57; 1915, lxii, 805.

[10] Aschoff, L., *Med. Klin.*, 1915, xi, 798.

[11] von Chiari, R. F., *Wien. klin. Woch.*, 1917, xxx, 1479.

[12] Löwy, O., *Wien. klin. Woch.*, 1916, xxix, 547.

The kidney, adrenals, liver, and testicles revealed no remarkable changes other than the characteristic microscopic nodular formation and the vascular changes. The lesions in these organs correspond to those found in man, in the kidney by Aschoff,[10] in the adrenals by Nicol,[4] in the liver by Ceelen,[3, 13] and in the testicles by Schmorl.[14]

To summarize the results of the experiments described above, it is shown that the blood of the patient at the Quarantine Station induced in guinea pigs a characteristic febrile reaction of from 4 to 8 days duration, preceded by an average incubation period of 9 days. The disease of which this is the expression could be transmitted through at least nine successive animals. Furthermore, the guinea pigs killed during the height of the reaction revealed on microscopic study of the organs constant pathological changes similar to those found by several investigators of typhus fever of man in Europe and Mexico, and of the experimental disease of the same animal.

In these respects, the experimental disease which we have induced in guinea pigs corresponds to that set up in the same species with the human and louse strains of the Polish virus.

Immunity Experiments.

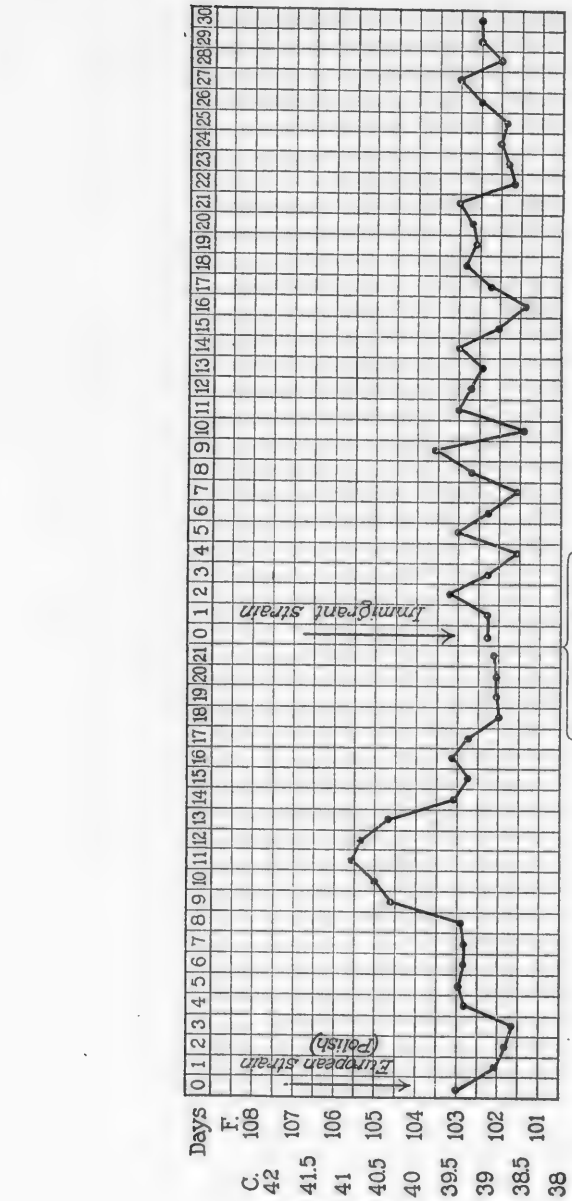
Additional evidence in establishing the identity of the experimental disease in guinea pigs induced with the blood of the immigrant with the disease propagated in these animals with the Polish virus, is afforded by means of the immunity reactions. The study on immunity was made from these view-points: (*a*) the specific immunizing effect of the virus derived originally from the blood of the immigrant when followed by subsequent injections of the active agent of the same disease, and (*b*) cross-immunity between this disease and that of guinea pigs originally infected with the Polish typhus fever virus of man and lice.

The following protocols are illustrative.

Protocol 2.—Guinea Pig B was injected intraperitoneally with 3 cc. of blood from Guinea Pig A, the fourth passage of the experimental disease in this species, derived originally from the blood of the immigrant. After an incubation period

[13] Ceelen, W., *Med. Klin.*, 1916, xii, 402.

[14] Schmorl, C. G., quoted by Nicol.[4]



TEXT-FIG. 2. This guinea pig, after reacting typically to an intraperitoneal injection of the Polish typhus fever virus, human strain, remained immune to a second injection of the immigrant disease.

of 9 days, Guinea Pig B showed a febrile reaction lasting for 6 days; thereafter the temperature was normal. A control guinea pig, injected similarly, reacted with a rise of temperature after 9 days incubation and was killed on the 1st day of the fever. It showed the typical pathological picture already described. Guinea Pig B after 6 days of normal temperature was reinjected intraperitoneally with 3 cc. of the blood from an animal in the sixth passage of the experimental disease. There was no reaction during a period of observation lasting 1 month. A control animal was inoculated at the time of the second injection of Guinea Pig B with similar material. This animal reacted characteristically after an incubation period of 10 days.

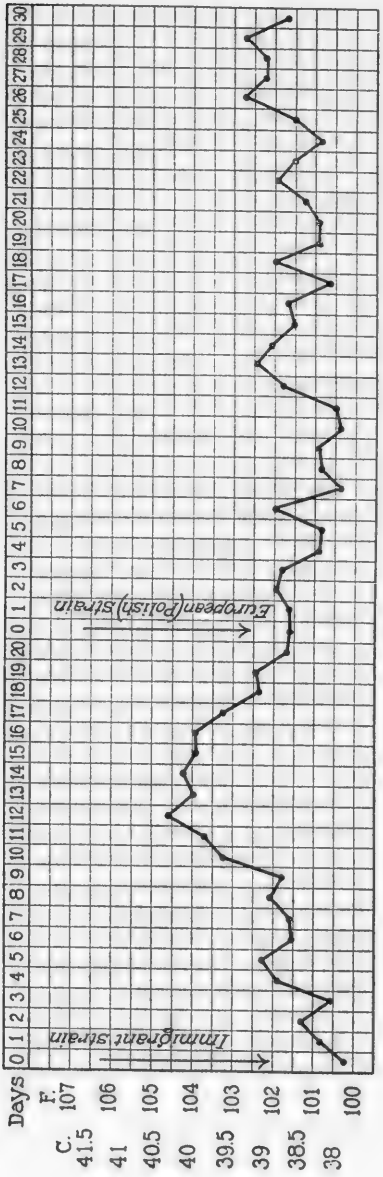
This experiment indicates that after an attack of the disease induced in guinea pigs with the blood of the immigrant, the animal is refractory to subsequent injections of active blood derived through passage from the same patient.

Protocol 3.—The following experiments were controlled in that whenever active blood was employed, it was injected into control animals under the same conditions as the test animal, to determine its clinical and pathological action.

Guinea Pig A (Text-fig. 2) was injected intraperitoneally with 3 cc. of blood from a guinea pig in the nineteenth passage of the experimental disease induced with the Polish strain of typhus virus. After an incubation period of 7 days a rise in temperature occurred, lasting for 5 days. Thereafter the animal showed normal temperature for 19 days, after which it was reinoculated with 3 cc. of blood from a guinea pig in the third passage of the experimental disease induced originally with the blood of the immigrant. There was no effect during a period of observation lasting 1 month.

Guinea Pig B was inoculated similarly, but with the eighteenth passage of the Polish typhus virus, louse strain. A 6 day fever following an 11 day incubation period resulted. 2 days after the fever subsided, the animal was reinjected with 3 cc. of blood from a guinea pig in the second passage of the experimental disease induced with the blood of the immigrant. No reaction occurred although the animal was kept under observation over a month.

Guinea Pigs C (Text-fig. 3) and D had reacted characteristically to injections of blood from guinea pigs in the fourth and fifth passages, respectively, of the experimental disease induced with the blood of the immigrant. Guinea Pig C, 4 days after the fever subsided, was reinjected with the blood from a guinea pig in the twenty-sixth passage of the Polish typhus virus, human strain; Guinea Pig D, 3 days after the fever subsided, with the blood of the twenty-fifth passage of the Polish typhus virus, louse strain. Neither animal was affected during a period of observation of over 1 month.



TEXT-FIG. 3. This guinea pig, after reacting typically to an intraperitoneal injection of the blood from a guinea pig in the fourth passage of the disease originally derived from the immigrant's blood, remained immune to a second injection of Polish typhus fever virus, human strain.

Protocol 3 shows that guinea pigs reacting characteristically to the known strains of typhus virus originally derived from a typhus fever patient in Poland, and from typhus-infected lice collected in that country, fail to respond to a subsequent injection of active blood from guinea pigs with the experimental disease set up with the blood from the Czecho-Slovakian immigrant at the New York Quarantine Station. Conversely, guinea pigs reacting typically to the injections of blood from guinea pigs in the line of transmission of the experimental disease induced with the immigrant's blood, are refractory to later injections of blood containing either human or louse strains of the Polish typhus fever virus.

DISCUSSION.

The experimental disease induced in guinea pigs with the human and louse strains of the Polish typhus virus is characterized by the following conditions: (*a*) Transmissibility of the virus from patient to guinea pig and from guinea pig to guinea pig indefinitely. (*b*) Specific pathology of the affected animals during the height of the reaction. The macroscopic examination reveals no changes in the organs with the exception of enlargement of the spleen[15] and a petechial rash in the deeper layers of the skin. The lesions in the different organs are demonstrable by histological study and consist of a particular vascular change, especially about the blood vessels of the brain. (*c*) Absence of concomitant or secondary infections by ordinary bacteria (pneumonia, peritonitis, abscesses, pseudotuberculosis, or other diseases, or the presence of these bacteria in the blood). (*d*) Specific immunity reactions. A guinea pig after reacting to an

[15] It has been stated that the organs in man (Nicol[4]) and in the guinea pig(Doerr[5]) show no macroscopic lesions whatever. But that the spleen is definitely enlarged has been our experience and this is confirmed by the following reports, both in regard to typhus fever in man and experimental typhus fever in animals: Ricketts, H. T., and Wilder, R. M., *J. Am. Med. Assn.*, 1910, liv, 463; Curschmann, H., Typhoid and typhus fever, Philadelphia, 1902, 526; Plotz, H., Olitsky, P. K., and Baehr, G., *J. Infect. Dis.*, 1915, xvii, 1; Nicolle, C., and Blaizot, L., *Arch. Inst. Pasteur Tunis*, 1916, ix, 127; Grzywo-Dabrowski, W., *Virchows Arch. path. Anat.*, 1918, ccxxv, 299; Danielopolu, D., Le typhus exanthematique, Bucharest, 1919, 222; Shattuck, G. C., Typhus fever with particular reference to the Serbian epidemic, Cambridge, 1920, 111, and others.

injection of the virus remains immune to a subsequent inoculation of the typhus fever virus.

It appears then that the blood from the immigrant could induce in guinea pigs a similar experimental disease, for, as will be noted in the above experiments, it is characterized by (*a*) transmissibility, (*b*) particular pathological effects, (*c*) absence of concomitant or secondary infections by ordinary bacteria, and (*d*) specific immunity reactions. From the experiments on immunity we infer that the strain of virus inducing the typhus fever occurring last year in Warsaw, Poland, in epidemic form, and that inducing the disease in immigrants this year at the Port of New York, are identical.

CONCLUSION.

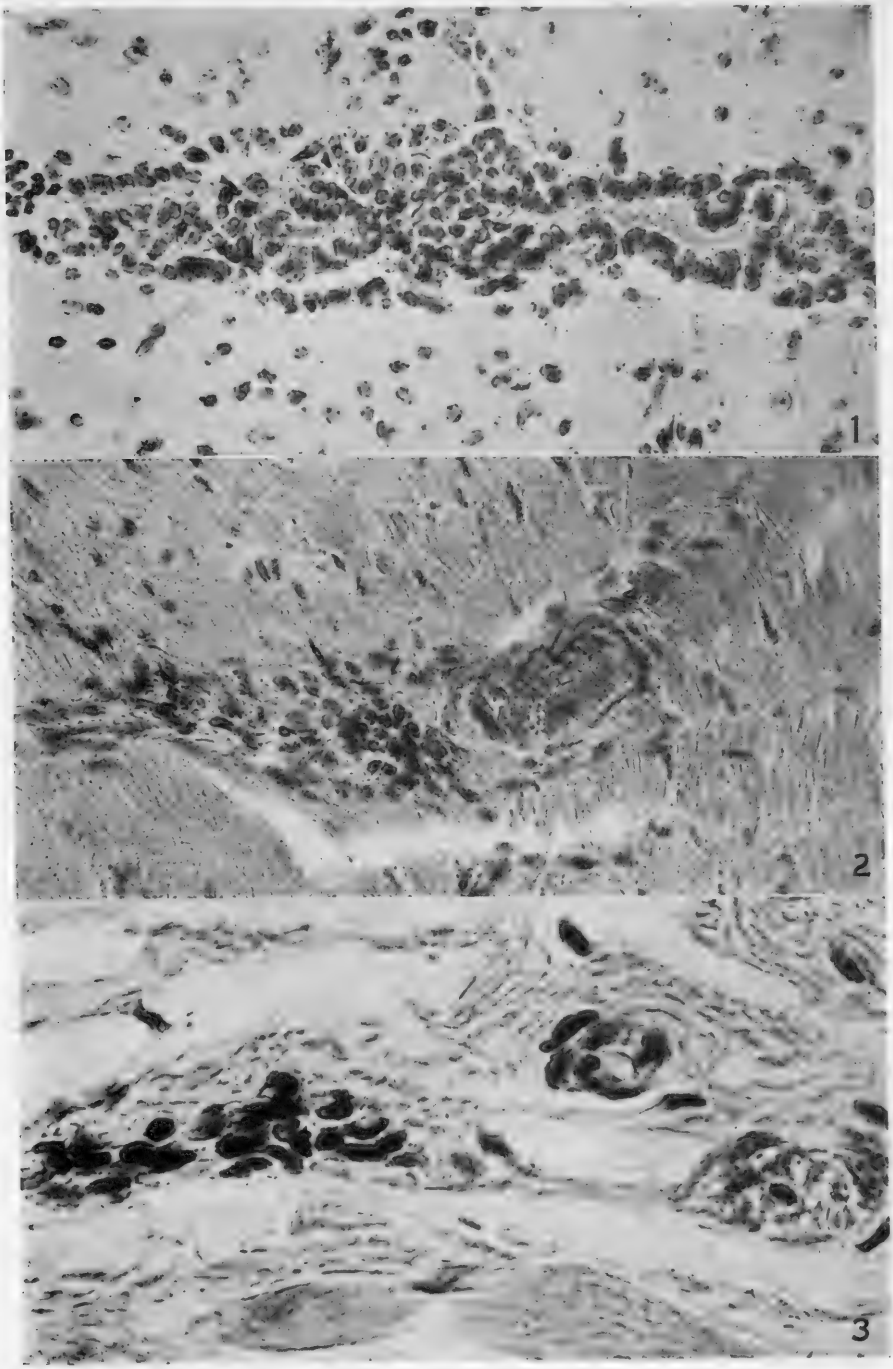
It is experimentally demonstrated in the guinea pig that the blood from an infected Czecho-Slovakian arriving at the Port of New York from Italy contained the virus of typhus fever and that this strain is identical with the epidemic virus present in man and louse in Poland.

EXPLANATION OF PLATE 25.

FIG. 1. Section of brain of guinea pig experimentally infected with the blood from the immigrant. The vascular lesions are shown in detail. There are circumscribed proliferation and accumulation of macrophages and leucocytes about an arteriole, and at one point, a *periarteriolitis nodosa*. × 400.

FIG. 2. Section of heart muscle from the same guinea pig. The vascular lesions are shown in detail. They resemble those found in the brain and demonstrated in Fig. 1. × 400.

FIG. 3. Section of the corium of the skin from a guinea pig experimentally infected with the blood from the immigrant (fifth guinea pig passage). The circumscribed accumulation of macrophages and leucocytes close to vessels, which show proliferation and swelling of the endothelial cells, is noteworthy. A thrombus is seen in one of the vessels. × 1,000.



(Olitsky: Typhus fever among recent immigrants.)

EXPERIMENTAL STUDIES ON INFLAMMATION.

I. The Influence of Chemicals upon the Chemotaxis of Leucocytes in Vitro.

By ELIZABETH PAULINE WOLF.

(From the Department of Pathology of the University of Chicago and the Otho S. A. Sprague Memorial Institute, Chicago.)

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Inflammatory processes usually, if not always, are the result of reaction to chemical substances. Some substances cause severe reactions when in very low concentrations, while others, even in high concentrations, cause little or no inflammation. As far as we can learn, no systematic study has ever been made to determine what chemical, physical, or physiochemical properties a substance must have in order that it may incite inflammation, or that it may be unable to incite inflammation. Can definite laws be discerned governing the capacity of chemical substances to stimulate inflammatory reactions? Is it possible to predict from the chemical or physical properties of a substance its capacity for inflammatory stimulation? Such information, if it can be obtained, should be of great value in chemotherapeutic work, since it might be applied to reducing the inflammatory reaction produced by drugs that must be injected into the tissues, and hence this study was undertaken at the suggestion of Dr. H. Gideon Wells as part of the program of chemotherapeutic investigation that is being conducted in the Otho S. A. Sprague Memorial Institute.

In studying this problem it is necessary to consider the several components of inflammatory reaction; namely, vascular changes, migration of cells and fluids, cell injury, and cell proliferation. In this paper will be considered only one phase of the work, the influence of chemicals upon the chemotaxis of leucocytes *in vitro*, a subject which has already received not a little investigation.

Most of the literature on this subject has been fully reviewed by Wells,[1] who has discussed the subject from the earliest studies of Engelmann who worked with protozoa in 1881, up to the time of Jacques Loeb, who worked with chemical tropisms. This may be briefly summarized in the following lists of the substances supposedly positive, negative, and neutral as chemotactic agents for leucocytes.

Various authors have stated that the strongly positively chemotactic substances are: sterilized and living cultures of bacteria, whether pathogenic or non-pathogenic; papayotin or papain (in rabbits) (Gabritchevsky[2]); carbolglycerol extracts of different viscera and tissues; egg albumin, gelatin, peptone, and alkali albuminate; metallic copper, iron, mercury, and their salts;[3] and substances soluble in lipoids in extreme dilution (Hamburger[4]).

Among the supposedly negatively chemotactic substances the following have been mentioned by various authors: concentrated solutions of sodium and potassium salts; lactic acid in all concentrations; quinine (0.5 per cent); alcohol (10 per cent); chloroform, in watery solution; jequirity (2 per cent, passed through a Chamberland filter); glycerol (10 to 1 per cent); bile; *B. choleræ gallinarum* (Gabritchevsky); mercury perchloride, cholesterol, arsenic, antipyrine, acetic acid; concentrated solutions of calcium, sodium, and potassium salts; alcohol in over 10 per cent concentration, chloroform, bile, glycerol in concentrations of 1 to 10 per cent; and atophan, or phenylcinchonic acid.

Among the neutral or indifferent agents have been mentioned water; calcium and sodium salts in concentrations of 0.1 to 1 per cent; phenol; phlorhizin; papayotin (in frogs); glycogen; peptone; blood; hemoglobin; uric acid up to 2 per cent; and bouillon.

These are only tentative statements, for many contradictory results have been obtained, and it is almost impossible to avoid some experimental errors.

The most important of the later work has been done by Hamburger, and his coworkers, on phagocytosis. This bears a close relation to chemotaxis and must be considered at the same time as chemotaxis. Hamburger[4] has shown that "calcium *per se*," as a substance causes a great rise in phagocytosis; it seems to be the only substance acting in this way, apparently aiding chemotaxis. He has investigated this problem from two view-points, *in vivo* and *in vitro* work both being done. In the *in vitro* work, horse leucocytes from citrated serum were transferred to different media and carbon particles added. The

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[1] Wells, H. G., Chemical pathology, Philadelphia, 4th edition, 1920, Chapter XI.

[2] Gabritchevsky, G., *Ann. Inst. Pasteur*, 1890, iv, 346.

[3] Bloch, G., *Centr. allg. Path. u. path. Anat.*, 1896, vii, 785.

[4] Hamburger, H. J., Physikalisch-chemische Untersuchungen über Phagozyten. Ihre Bedeutung von allgemein biologischem und pathologischem Gesichtspunkt, Wiesbaden, 1912.

percentage of these particles taken up by the leucocytes was used as a measure of the degree of phagocytosis. The *in vivo* work was done by injecting calcium-free and calcium-containing cultures of bacteria (*B. coli*) under the skin in capillary tubes, and then comparing the size of the resulting leucocyte columns in the calcium-free and calcium-containing cultures. Besides the work on calcium, Hamburger has tested many organic and inorganic compounds. He found that, under the conditions of his experiments, hemoglobin had no bad effects on phagocytic activity no matter what concentrations were employed. Collargol and other colloidal silver compounds had no effect up to 0.02 per cent, but above that concentration these compounds were injurious and the injury increased in proportion to the concentration. Quinine was injurious to leucocytes, seeming to act upon the cells themselves. Of the halogens, iodine is more poisonous than bromine; fluorine is a strong poison; iodoform increases phagocytosis; mercury, strontium, and barium are indifferent. All fat solvents in small amounts increase phagocytosis; those used were chloroform, ether, chloral, benzene, camphor, some fatty acids, and turpentine. Citrate and sulfite ions act as a poison, preventing any phagocytosis. Oxygen acts like apnea, preventing phagocytosis; carbon dioxide increases phagocytosis. Potassium cyanide has about the same action as carbon dioxide, an increase followed by a decided decrease in phagocytic activities, and then death of the cells.

Method.

In my work, Wright's method has been employed.[5] Equal volumes of blood must be disposed over equal areas of surface, and come into contact with equal concentrations of the desired reagent throughout. This may be done in the following ways: The first method is to paint hot paraffin containing the desired reagent upon glass slides until 1 mm. thick, then punch circles in the plastic paraffin with a sharp cork-borer, and prize out the discs and clean the glass floor with xylol. The second and preferable method is to use agar in the place of paraffin. Here different concentrations of the drugs desired are mixed with 2 per cent salt-free water solution of agar, then cast into tablets 1 mm. thick, in molds constructed of a pair of glass slides held apart at the ends by pieces of slides 1 mm. thick. The slide which forms the lid of the mold is pushed aside, while the agar is poured in, then brought into position. When this melted agar has set the lateral supports and the lid are removed and circles are cut in the agar with a sharp cork-borer, the discs removed,

[5] Wright, A. E., *Lancet*, 1918, i, 129.

and the cells thus obtained placed upon slabs of 2 per cent salt-free water solution of agar, or upon slabs containing the same concentration of the reagent as contained in the cell. In this way, a window is formed, with the floor and sides of agar containing the desired concentration of the reagent. Two different forms are used. In one, the floor consists of 2 per cent salt-free water solution of agar, the sides of the 2 per cent solution of agar containing the desired reagent. In the other, the floor and sides of the cell contain the same concentrations of the substances to be tested. These different cells are then placed upon a glass slide so that each slide contains from eight to twelve cells, each one of which has a different concentration of the reagent. On each slide there is a control cell, the floor and sides being made of the 2 per cent salt-free water solution of agar.

The slides containing the agar slabs were kept in moist chambers made of Petri dishes whose bottom and sides were lined with moist cotton, so that no water would be lost by evaporation from the agar, thus changing the concentrations. The Petri dishes were kept in warm water, the slides thus being heated to 37°C. and kept at this heat so that the temperature would be the same in each window when the blood was run in and all the time after pouring. Equal amounts of blood were then run into the cells thus made. A capillary pipette was filled with blood as soon as the vein was cut, and 2 drops of this uncoagulated blood were allowed to drop into each window immediately so that there would be no chance of clotting. All the windows were filled at one time so that there would be no differences in the blood itself to be taken into consideration. The dishes containing the blood-filled windows on the slabs of agar were put in the incubator and kept at 37°C. for 45 minutes. After incubation, the clots of blood were washed off with warm sodium chloride solution, 0.9 per cent sodium chloride in distilled water, kept at 37°. If the clots were extremely adherent to the sides, the edges were loosened by touching them gently with a probe, or lifted out with fine forceps. A solution of saturated mercuric chloride could then be poured into the windows to fix the leucocytes if desired. The number of leucocytes adhering to the sides and the floor were then counted under the low power (No. 3) of the microscope.

In this work repeated duplicate tests were made with the blood of the same animal. The number of cells migrating into the agar was always the same within the limits of experimental error, the difference never being more than 25 to 50 cells out of a total of 2,000 to 2,500. The same percentage of the total number of cells always adhered to the control slide; *i.e.*, about 4 per cent. The exact number varied with the degree of leucocytosis but the relative number was always approximately the same.

From 0.0001 to 10 per cent molecular concentrations of the different salts were employed, so that the range of chemotaxis might be studied in relation to the per cent of the salt itself, as well as the positive and negative ions composing the salt, as, for example, NaCl as a salt and Na as an ion as compared with Na in Na_2CO_3, or Na_2S_4O, and Cl as an ion as compared with Cl in KCl or $CaCl_2$, etc. In some cases a high concentration showed a marked negative chemotaxis, while a low concentration showed a positive one. Whenever possible, neutral or nearly neutral substances were used, so that the reaction would not interfere with the chemotaxis. If, however, the chemical had an acid or alkaline reaction strong enough to give irregular and peculiar results, sodium phosphate as the tri-, di-, or monobasic salt was added in just sufficient concentrations to give a neutral reaction with phenolsulfonephthalein (pH = 7.7), and the results were then compared with that of the original chemical alone.

For each substance used, at least four different tests were made with human blood, five with rabbit blood, and two or three, when possible, with guinea pig and dog blood, so that the mean of the several bloods of each species might be used as the final count, providing there was little individual variation. Since the degree of leucopenia or leucocytosis made a great difference in the exact number of leucocytes adhering, though not the relative number, as a routine matter white cell counts were made on the blood of all animals every week, since it was found that in health there is little variation in that length of time. It was found that human blood varied markedly before and after eating, so certain times after eating were chosen for drawing blood when possible, usually $2\frac{1}{2}$ to 3 hours after a meal.

An interesting observation is that immediately after eating, within 30 minutes, the leucocytes stick much more readily to the

agar than at other times. This holds good for all the species tried. This has been noted for leucocytes on glass slides by several investigators. Cohnheim, among others, mentions the difference in behavior of leucocytes at different times of the day, but the reasons for this have never been shown.

The experimental animals were kept from food for about 3 hours before use. When possible the same animals were used with the different drugs. It was found that with a 2 per cent solution of salt-free agar about 4 per cent of the leucocytes would always adhere to the agar. This was constant with the blood of all the animals employed.

The following chemicals were used:

1. Calcium salts, as the chloride, carbonate, tartrate, citrate, lactate, sulfate, cyanide, and cinnamate.
2. Sodium salts, as the chloride, carbonate, tartrate, citrate, lactate, sulfate, cinnamate, and tri-, di-, and monobasic phosphates.
3. Potassium chloride, acid carbonate, and acid phosphate.
4. Barium sulfate and sulfide.
5. Strontium chloride.
6. Magnesium sulfate, tartrate, citrate, and these compounds mixed with strontium salts.
7. Mercury chloride, nitrate, subsalicylate, and iodate.
8. Organic acids, as oleic, lactic, uric, and nucleinic.
9. Amino-acids and amines, as tyrosine, histidine, glycine, alanine, glutamic acid, aminovalerianic acid, tyramine, and histamine.
10. Alkaloids, as morphine and morphine sulfate, hydrochloride, nitrate, and citrate; codeine and codeine hydrochloride; heroine and heroine hydrochloride; caffeine sulfate, quinine sulfate, strychnine sulfate, and brucine sulfate.
11. Miscellaneous substances, as papayotin, or papain, urea, urethane, creatinine, ethyl alcohol, chloral hydrate, cantharidinum, aspirin (acetyl-salicylic acid), chloretone, glucose, "mustard gas" (dichloroethyl sulfide) "parazol" (dinitrodichlorobenzene), olive oil, scarlet R (purified), turpentine, veronal (diethylmalonylurea), yeast vitamine (Harris), acetanilide, and ether.

EXPERIMENTAL.

Calcium Salts.

All calcium salts were positively chemotactic for leucocytes except calcium citrate. This was neutral in a 1 per cent molecular concen-

tration, but all other concentrations were negatively chemotactic, the citrate ion overpowering the calcium ion.

Calcium chloride showed a marked increase in the number of cells adhering to the agar with which it was combined, as compared with the control. When there was more than 2 per cent molecular concentration, there was a precipitate through which the light could not penetrate, so that the cells could not be counted when the floor was composed of agar containing the same concentration of the salt as the margins.

An illustrative series of experiments with calcium chloride is given in detail below. The figures in the top row are the percentage of calcium chloride contained in the water agar solution of each cell, and this represents the molar or gram-molecular concentration of calcium in calcium chloride. Thus in the case of a 5 per cent solution 2.75 gm. of calcium chloride would be used with 20 cc. of a 2 per cent solution of water agar, so that there would be 1 gm. of calcium present in 20 cc. of the agar solution. An exact count was made of the number of cells migrating into the agar and adhering, after the clot had been removed and the window and floor of the cell washed with normal salt solution, and this is recorded in each of the remaining rows. Each row gives results obtained with a separate sample of blood. The lowest row of figures in each group is the mean of the other figures in that column.

In the second group of figures, from observations with the floor as well as the margins of the cell formed of agar plus the different concentrations of calcium chloride, it will be seen that many more cells adhere in concentrations of 0.5 to 2 per cent than when only the margins of the cell contained calcium chloride. When frozen sections were made of these different agar plates it was found, after examination of the serial sections, that the leucocytes migrated into the agar to the depth of 0.68 to 0.84 mm. when calcium chloride was combined with the agar, while the migration into the plain agar occurred only to the depth of 0.22 to 0.47 mm. when the margins contained the calcium chloride.

Calcium Chloride Combined with Agar. Floor of 2 Per Cent Plain Agar. Human Blood.

	5 per cent.	3 per cent.	2 per cent.	5 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.08 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	Control.
	2,108	3,149	2,508	3,097	3,146	3,564	4,036	4,587	4,562	3,736	2,836	2,564	2,573	1,892
	1,872	2,569	3,095	2,764	3,593	4,095	4,539	3,842	4,594	3,874	2,893	2,482	2,531	1,936
	1,869	2,546	3,148	3,056	3,582	4,082	3,584	3,792	4,327	2,746	2,683	2,538	2,443	2,180
	2,304	2,492	3,345	3,543	4,726	5,238	5,098	5,070	4,956	4,086	3,082	2,775	2,132	2,038
	2,495	3,178	3,487	3,642	4,946	5,654	5,187	5,136	5,032	4,301	3,149	2,867	2,845	2,645
	1,657	3,482	3,542	5,035	5,692	5,237	5,109	5,192	5,017	4,785	3,376	2,850	2,742	2,543
Average	2,051	2,901	3,187	3,523	4,260	4,645	4,591	4,603	4,746	3,921	3,003	2,696	2,546	2,205

Floor of 2 Per Cent Agar Plus Calcium Chloride. Human Blood.

	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.08 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	Control.
	8,324	7,891	8,513	7,549	4,079	5,538	3,175	2,486	2,568	3,125	3,146
	5,876	5,683	5,962	5,872	3,762	4,163	2,104	1,973	2,015	2,047	1,985
	5,769	5,468	5,870	5,637	3,652	4,075	2,346	2,074	2,016	2,168	2,042
	5,678	5,469	6,085	5,364	3,869	4,197	2,834	1,985	2,175	2,068	2,164
	5,697	5,683	6,197	5,639	3,749	4,329	2,096	2,438	1,986	2,157	2,352
	6,107	5,789	5,985	6,097	3,578	4,097	2,189	2,357	2,439	2,146	2,458
Average	6,242	5,997	6,452	6,115	3,761	4,396	2,457	2,235	2,206	2,260	2,356

Calcium tartrate was slightly positively chemotactic, there being an increase of 25 per cent in the number of cells adhering at 0.25 per cent molecular concentration, as compared with the control.

Calcium lactate was slightly positively chemotactic despite the negative influence of the lactate ion, there being an increase of 30 per cent over the control at 0.1 to 0.05 per cent molecular concentration.

Calcium cyanide was slightly positively chemotactic when the floor of the cell consisted of plain water solution of agar and the sides of the window consisted of 2 per cent water solution of agar plus calcium cyanide. When both the floor and the sides of the

cell were made of the agar containing the cyanide, there was a slightly negative chemotaxis. This may possibly be explained on the basis that calcium cyanide, except in minute quantities, is highly toxic, and only in these small quantities does the chemotactic influence of the calcium overcome the cyanide poisoning. It has been because of these variations in action that the plain slab and the salt-containing slab have been used in every case. This will be referred to later. The average figures obtained in five experiments with calcium cyanide and human blood are given below:

Calcium Cyanide.

1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
3,614	3,548	4,018	3,564	3,532	3,147	2,591	1,403	2,017
—	—	2,018	2,136	547	536	241	218	2,106

In all the following tables, the first row of figures will be the average of five experiments for those cells in which the floor of the cell consisted of salt-free water solution of agar, the lower rows of figures will be for those cells in which both the floor and walls were composed of agar containing the tested reagents. Because of space restriction only a very small proportion of the actual results are published, for the purpose of illustrating the procedure.

Calcium carbonate, calcium sulfate, and calcium cinnamate were all somewhat positively chemotactic and varied very little from each other. The greatest number of leucocytes adhering was when the molecular concentration was 0.25 per cent, there being an increase of 50 per cent over the control.

Calcium citrate was negatively chemotactic in all concentrations over or below 1 per cent, as mentioned previously. A typical set of average figures for human blood with calcium citrate follows:

Calcium Citrate.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
1,507	2,464	3,092	2,573	2,798	1,549	1,485	1,502	3,017
1,673	2,793	3,052	2,489	2,538	1,537	1,426	1,507	3,164

Sodium Salts.

The sodium salts had no constant action that depends on the sodium ion *per se*. Sodium chloride was positively chemotactic at 1, 0.8, and 0.75 per cent molecular concentrations, nearly twice as many leucocytes adhering as in the control slides with the blood of the same animal. In more than 5 per cent molecular concentration there was a negative chemotaxis, only two-thirds to one-half as many cells adhering as in the control slides. These figures applied to all blood, human, rabbit, dog, and guinea pig. A typical set of figures obtained in a series of experiments with sodium chloride is shown below:

Sodium Chloride.

7.5 per cent.	5 per cent.	4 per cent.	3 per cent	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	Control.
1,042	1,965	2,386	2,576	3,091	4,027	3,957	2,538	2,273	2,015	1,869
1,036	1,538	2,069	2,537	3,096	4,586	4,097	2,437	2,097	2,175	2,013

Sodium sulfate was neutral in regard to chemotaxis in all concentrations below 4 per cent molecular concentration for all animals. Above this concentration, it was negatively chemotactic. Sodium carbonate was neutral in almost all concentrations, although with from 0.8 to 1.2 per cent molecular concentration there was a slight positive chemotaxis. Sodium tartrate was neutral for almost all concentrations. Sodium citrate was neutral in all concentrations below 0.1 per cent; above this it was negatively chemotactic for human blood; for rabbit blood there was a slight degree of negative chemotaxis for molecular concentrations above 0.5 per cent.

Sodium Citrate.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
Human blood.							
1,579	1,468	1,452	1,792	2,247	2,296	2,158	2,375
1,538	1,396	1,425	1,658	2,153	2,153	2,096	2,436
Rabbit blood.							
2,548	2,459	2,197	2,987	3,084	3,178	3,097	2,976
2,356	2,351	2,146	2,857	2,967	3,089	3,096	2,963

Sodium lactate was negatively chemotactic in all concentrations for human blood, but with rabbit blood there was no negative chemotaxis, and with the blood of three of the five animals, there was an increase of about 25 per cent in the number of leucocytes adhering at 0.05 to 0.1 per cent molecular concentration.

Sodium cinnamate was somewhat positively chemotactic in molecular concentrations of 2 per cent and over. In lower concentrations it was neutral. This held true for the blood of man, rabbits, and dogs.

Sodium phosphate was positively chemotactic in varying degrees as the tri-, di-, and monobasic salt.

In the work on sodium phosphate, advantage was taken of the fact that pneumonia patients who had received large doses of sodium phosphate either by mouth or intravenously showed an increased number of leucocytes migrating into the agar containing sodium phosphate or calcium chloride. A normal person was used as control and the blood tested at intervals after the same doses of sodium phosphate. It was found that, alike in pneumonia and in health, many more cells migrated into agar containing Na_2HPO_4 or $CaCl_2$ than in the case of those people who had not received the sodium phosphate medication. A typical set of figures is shown below for the blood of patients who had received large doses of sodium phosphate, when tested with Na_2HPO_4 agar.

Na_2HPO_4.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
Pneumonia blood.								
6,182	7,023	7,147	10,862	7,046	7,136	7,563	7,014	5,409
11,427	15,356	15,876	18,973	14,899	14,992	7,642	7,924	5,311
Normal human blood.								
4,847	4,939	4,883	4,439	4,872	4,976	4,895	3,869	2,197
6,436	6,854	6,973	9,037	6,853	6,693	3,246	3,386	2,201

The blood of all animals showed a positive chemotaxis with Na_3PO_4 in molecular concentrations of 0.1 to 2 per cent; in concentrations

over 2 per cent there was a slight negative chemotaxis, probably due to the alkaline reaction.

With both NaH_2PO_4 and Na_2HPO_4 marked positive chemotaxis was produced, as shown by the two following tables:

NaH_2PO_4 with Normal Human Blood.

4 per cent.	2 per cent.	1 per cent.	0.8 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	Control.
4,387	4,097	4,028	4,538	4,538	3,327	3,107	2,145	2,047
4,038	6,359	6,109	7,537	7,634	6,094	6,024	2,214	2,046

Na_2HPO_4 with Normal Human Blood.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	Control.
10,847	5,432	7,096	7,756	4,483	3,224	2,875	2,846
9,856	8,951	9,943	8,926	6,453	4,954	4,493	2,893

When to calcium chloride NaH_2PO_4 was added, there was a marked increase in the number of cells adhering. There seemed to be a synergistic action between sodium phosphate and calcium chloride, the increase in the number of cells being greater than could be accounted for by the addition of the cells adhering with either of the two chemicals alone.

$CaCl_2$ Plus NaH_2PO_4.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	Control.
10,752	11,765	10,967	9,870	10,236	9,846	3,245	2,346
13,257	14,735	11,364	12,453	13,247	9,364	6,325	2,433

Potassium Salts.

All potassium salts were negatively chemotactic for all blood of all animals employed when concentrations over 0.01 per cent (molecular concentration) were used. This held true for potassium chloride, potassium acid carbonate, and potassium acid phosphate. In molecular concentration below 0.01 per cent there was a neutral response with all blood, human, rabbit, dog, and guinea pig.

Barium Salts.

Barium sulfide was slightly negatively chemotactic in molecular concentration over 0.05 per cent. Barium sulfate was slightly positively chemotactic.

Strontium Chloride.

Strontium chloride was slightly positively chemotactic, but strontium chloride with equal molecular concentrations of magnesium sulfate gave a great increase in the number of cells adhering to the agar. Here again the action seemed synergistic, since neither alone gave marked reaction, and many more cells adhered to the agar than could be accounted for by mere addition of the cells emerging when both salts were used.

Magnesium Salts.

Magnesium salts acted in the same way as sodium salts in regard to chemotaxis, this seeming to depend largely on the negative ion attached to the magnesium. Magnesium sulfate was neutral as regards chemotaxis in all concentrations for all blood. Magnesium tartrate was slightly negatively chemotactic in concentrations over 1 per cent, but neutral in all concentrations below this for all blood used. Magnesium citrate was slightly negatively chemotactic for all blood but more so for rabbit than for human blood.

Mercury Salts.

All mercury salts behaved about the same in regard to chemotaxis. They seemed to fix the blood before the cells could migrate into the agar, so that only a few could be counted. The clot formed was hard and brittle. This apparent negative chemotaxis of mercury salts is really a fixation of proteins and not a chemotaxis of any kind. For these reasons, the action of these compounds must be studied in some other way to determine the degree of chemotaxis they possess.

Organic Acids.

Oleic acid was positively chemotactic. This substance was used with the paraffin method so that the acid would dissolve quickly

throughout. Since, however, only the sides of the cell thus formed contained the reagent, this was unsatisfactory. For this reason, an emulsion of oleic acid with agar was made and then cooled and hardened rapidly over ice so that the floor and sides of the cell thus formed would contain the same concentration of the reagent. There was an increase of 100 per cent in the number of cells adhering at 0.5 per cent concentration over the control; in concentrations over 1 per cent there was no chemotactic effect, about the same number of cells adhering as in the control. The sodium salt of this acid showed only a very slight increase in the number of cells adhering at 0.1 per cent molecular concentration; above this there was a slightly negative chemotaxis.

Lactic acid was negatively chemotactic. This may have been due in part to the hydrogen ion concentration, but the sodium salt was also negatively chemotactic although not so markedly so as the pure acid. There were only half as many cells adhering with lactic acid in concentrations above 0.05 per cent as there were in the control.

Uric acid was slightly negatively chemotactic in molecular concentrations above 0.5 per cent; below this concentration it had no effect.

Nucleinic acid made from yeast was positively chemotactic in molecular concentrations of 0.0005 to 0.00001 per cent or percentage concentrations of 0.0065 to 0.00013; when the acidity was overcome by by the addition of a slight amount of sodium hydroxide (hydrogen ion concentration pH = 7.7), there was an increase in the number of cells adhering. With the free acid there were two-thirds more cells adhering at 0.0025 per cent molecular concentration (0.0325 per cent by weight) than in the control. With the addition of the sodium hydroxide there were twice as many cells adhering as in the control.

Amino-Acids and Amines.

Glycine and alanine were slightly positively chemotactic, alanine being somewhat more so than glycine. At 4 per cent concentration there were about three times as many cells adhering as in the control when glycine was used, while with alanine there were about three and one-half times as many cells adhering.

Tyrosine was somewhat positively chemotactic. There were about three times as many cells adhering with molecular concentrations of 0.0001 to 0.001 per cent as in the control. Tyramine, made synthetically,[6] acted most peculiarly. The cells clumped in such a way that it was impossible to count or even to estimate the number migrating into the agar with any degree of exactitude. This was repeated many times, the same thing occurring each time. No reason could be found for this action of tyramine. It suggested the appearance of a calcium cyanide clot, but differed in that the cells were much more firmly adherent to each other with tyramine than with calcium cyanide.

Histamine[7] was strongly positively chemotactic in molecular concentrations of over 0.000025 per cent (0.000055 per cent by weight), but the leucocytes of dog and human blood showed a greater degree of migration into the agar containing histamine than did the leucocytes of rabbit blood. One set of illustrative figures will be given for human, one for dog, and one for rabbit blood.

Histamine.

0.005 per cent.	0.00025 per cent.	0.0001 per cent.	0.00005 per cent.	0.000025 per cent.	0.00001 per cent.	0.000005 per cent.	0.0000025 per cent.	Control.
Human blood.								
7,021	6,538	5,028	2,537	3,159	1,138	1,274	1,485	1,407
7,183	6,983	9,894	1,093	4,963	2,915	1,019	1,857	1,396
Dog blood.								
5,379	5,027	2,473	1,532	2,036	1,463	3,527	4,037	3,016
7,037	7,239	7,635	6,528	6,017	3,012	5,172	5,187	3,423
Rabbit blood.								
4,507	4,017	3,012	1,093	1,004	1,025	1,028	857	1,046
—	4,321	3,294	993	3,653	1,946	1,956	1,845	1,243

Histidine from the same source showed a slight positive chemotaxis for all blood, human, dog, and rabbit. There was an increase of about

[6] The tyramine was made by Dr. Koessler and Dr. Hanke.

[7] This was also obtained from Dr. Koessler and Dr. Hanke.

25 per cent in the number of cells adhering at 0.005 per cent over the number adhering in the control window.

Glutamic acid was slightly positively chemotactic in concentrations of 0.0005 to 0.00001 per cent; when the acidity was overcome by the addition of a slight amount of sodium hydroxide, the solution being neutral to phenolphthalein (pH = 7.7), there was an increase in the number of cells adhering. When the free acid was used there was an increase of 100 per cent in the number of cells adhering with concentrations of 0.005 to 0.00001 per cent, while with the addition of sodium hydroxide the increase was 200 per cent over the control.

Aminovalerianic acid was positively chemotactic in all concentrations to about the same extent as glutamic acid. With the addition of sodium hydroxide there was also an increase in the number of cells adhering. A typical series for aminovalerianic acid and human blood is:

Aminovalerianic Acid.

0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	0.0001 per cent.	0.00005 per cent.	0.00001 per cent.	0.000005 per cent.	Control.
3,537	4,091	4,486	3,147	2,159	2,038	1,537	2,046	3,034	1,986
6,283	5,897	5,546	4,138	5,589	3,143	3.107	3,186	3,037	2,037
Aminovalerianic acid with NaOH added to neutrality.									
4,031	4,899	4,998	3,887	3,926	3,967	4,037	3,448	3,963	2,015
9,837	9,853	9,475	6,015	4,084	3,996	3,026	4,035	5,863	2,104

Alkaloids.

Morphine was slightly positively chemotactic for human and dog blood in most concentration for all morphine compounds used except the citrate. This is contrary to most of the work that has been done with morphine and its compounds, but after many repetitions the same result was obtained. There is much less increased migration of leucocytes with rabbit than with human blood. Morphine sulfate showed a slight but constant positive chemotaxis for human and dog blood in concentrations of 0.3 to 2 per cent, for rabbit blood in concentrations of 3 per cent. Morphine, morphine hydrochloride and nitrate,

all showed the same degree of chemotaxis. Morphine citrate was practically neutral in concentrations below 0.05 per cent, but above that it was negatively chemotactic.

Morphine Hydrochloride.

3 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
Human blood.										
	1,817	1,537	1,509	1,598	1,648	1,892	2,147	3,027	2,568	1,785
	2,047	5,538	5,018	5,537	5,536	5,023	4,321	4,572	4,012	1,825
Rabbit blood.										
3,018	1,016	1,546	1,512	1,478	1,443	1,976	2,314	2,215	2,415	2,648
4,001	1,192	2,032	1,472	2,351	2,511	1,897	1,998	2,322	2,451	2,771

Heroine and heroine compounds were neutral as regards chemotaxis. Codeine and codeine compounds were neutral in most concentrations, but in concentrations over 1 per cent there was a slight negative chemotaxis.

Caffeine sulfate was negatively chemotactic for all blood in all concentrations. Quinine sulfate, strychnine sulfate, and brucine sulfate were all negatively chemotactic for all blood in all concentrations.

Miscellaneous Substances.

Papayotin, or papain, was markedly negatively chemotactic for human blood in all concentrations. In fact, only 5 to 100 leucocytes adhered to the agar containing concentrations of papayotin over 0.05 per cent. With rabbit blood there was a remarkable difference, a positive chemotaxis being observed with all concentrations over 0.05 per cent. This decided chemotaxis for rabbit blood is mentioned by Gabritchevsky. Dog blood came midway between rabbit and human blood, while the blood of frogs varied with the temperature of the body; thus the winter frog blood was negatively chemotactic, the summer frog blood was positively chemotactic.

Papayotin.

4 per cent.	2 per cent.	1 per cent.	0.5 per cent.	0.1 per cent.	0.05 per cent.	0.01 per cent.	0.005 per cent.	Control.
Human blood.								
120	110	100	102	135	385	732	983	2,361
16	34	35	74	52	87	128	1,184	2,752
Rabbit blood.								
6,284	6,938	6,733	5,732	3,462	3,352	2,573	2,283	4,198
6,988	6,793	6,990	5,938	4,521	3,710	3,109	2,846	4,017

Urea, urethane, and creatinine were all negatively chemotactic for the blood of all animals, in molecular concentrations above 0.5 per cent; below this concentration they were neutral.

Ethyl alcohol was negatively chemotactic in all concentrations over 2 per cent, no matter whether the paraffin or agar method was used. In concentrations under 2 per cent, alcohol was neutral.

Cantharidin, or cantharidinum (U.S.P.), was positively chemotactic. Cantharidin was obtained from Chinese cantharides and the purified cantharidin was dissolved in 70 per cent alcohol and an emulsion made with agar. This emulsion was cooled rapidly over ice, then the windows were cut as in previous cases. The blood of different animals varied markedly towards cantharides. This was strikingly shown for rabbit blood. The rabbit seems to have a congenital tolerance for cantharidin, and rabbit leucocytes were little influenced by cantharidin, which was decidedly positively chemotactic for leucocytes from other species.

Cantharidin.

0.5 per cent.	0.25 per cent.	0.1 per cent.	0.05 per cent.	0.025 per cent.	0.01 per cent.	0.005 per cent.	0.001 per cent.	0.0005 per cent.	Control.
Human blood.									
5,987	3,863	3,993	7,946	5,124	2,136	1,872	2,044	2,115	2,109
1,928	6,937	4,837	8,123	5,923	1,927	1,764	2,942	2,012	2,213
Rabbit blood.									
6,001	4,336	4,118	2,119	2,139	2,003	1,947	3,024	3,642	3,798
1,125	2,453	1,362	2,774	3,632	3,587	1,176	3,531	3,346	3,631

It will be noticed that when the concentration of cantharidin was over 0.25 per cent, fewer cells migrated into the agar combined with cantharidin than into the plain agar with a margin of agar plus cantharidin. It is possible that strong concentrations of markedly chemotactic substances have a repelling action. This is shown more markedly in the case of rabbit blood, for here there seems to be a repelling action in all concentrations over 0.05 per cent in the cells with a cantharidin-containing floor.

Aspirin (acetyl-salicylic acid), acetanilide, and chloral hydrate were all negatively chemotactic in all concentrations for all blood. The reduction in the number of cells adhering varied from 20 to 60 per cent below the normal. Fewer cells adhered in concentrations over 0.5 per cent than below. At 4 per cent concentration, with all of these reagents, only about 40 per cent of the control number of leucocytes adhered.

Chloretone (Parke, Davis and Company) was slightly positively chemotactic in practically all concentrations, the increase varying from about 5 to 20 per cent over the control.

Glucose was neutral in all concentrations under 20 per cent; above that there was a very slight negative chemotaxis.

Mustard gas (dichloroethyl sulfide) was practically neutral in all concentrations. An emulsion was made of the sulfide in oil with agar and then cooled rapidly over ice. When the sulfide in oil was added to paraffin, more of the gas volatilized because the oil had to be added at a much higher temperature, due to the higher melting point of the paraffin than of the agar. With paraffin, also, the floor consisted of the plain glass so that the effects of the reagent could be seen only along the sides of the window thus made.

Parazol, or crude dinitrodichlorobenzene,[8] was somewhat positively chemotactic in concentrations under 1 per cent; over that it was negatively chemotactic.

Olive oil was neutral in all concentrations.

Scarlet R, purified, was slightly positively chemotactic in concentrations of 0.5 to 2 per cent, below that it was neutral.

[8] Concerning the toxicity and properties of parazol see Wells, H. G., *J. Ind. Hyg.*, 1920, ii, 247; Voegtlin, C., Livingston, A. E., and Hooper, C. W., *Bull. Hyg. Lab., U. S. P. H., No. 126*, 1920, 183.

Turpentine was slightly positively chemotactic in concentrations over 0.1 per cent; there was an increase of 50 per cent over the control at 0.5 per cent concentration.

Veronal was slightly negatively chemotactic in all concentrations over 0.5 per cent; below that it was about neutral.

Yeast vitamine (Harris) was slightly positively chemotactic in concentrations over 0.1 per cent. This may be due to the fact that many amino-acids and extractives are contained in this substance, and as they are positively chemotactic, anything in which they are found will show a certain degree of positive chemotaxis.

Ether was slightly positively chemotactic in concentrations over 0.1 per cent, when there was an increase of about 50 per cent over the control in the number of cells adhering.

DISCUSSION.

This new method of Wright's has been found very satisfactory for the determination of chemotaxis *in vitro*. It has been modified somewhat; *i.e.*, there is no need for calibrated capillary pipettes, as ordinary capillary pipettes may be used and 1 or 2 drops from the same pipette will give the same amount of blood. Cooling will cause changes in the reaction, so that it is necessary to keep the blood at 37°C. at all times. The hydrogen ion concentration is an important factor and this has been considered carefully.

This method is definitely quantitative. All steps may be carefully controlled, so that factors of error may be practically eliminated. Substances may be used with this method that have heretofore never been studied as regards their chemotactic powers; among these are cantharidin, histamine, mustard gas, parazol, and tyramine. The results obtained by this method with substances frequently studied, agree in practically all particulars with those obtained by other workers with different methods. It may also be employed as a method of determining the action of unknown drugs in producing the migration of leucocytes at the site of injection. The results obtained with sodium phosphate compounds, showing them to be strongly positively chemotactic and increasing the chemotaxis of the blood of those taking them either by mouth or intravenously, are

of interest in relation to recent work on the use of these salts in the treatment of many infections.

It has been found that the blood of different species of animals may react differently towards different drugs; thus, rabbit blood shows much less positive chemotaxis for cantharidin than human or dog blood. On the other hand, rabbit blood was much more markedly positively chemotactic for papayotin than human or dog blood. With histamine, human and dog blood showed more cells migrating into the agar than did rabbit blood. There seems to be no apparent underlying reason for this difference in action of the blood of different animals.

CONCLUSIONS.

1. Wright's method for the study of chemotaxis of leucocytes *in vitro*, slightly modified, has been found to be most satisfactory in the estimation of the degree of chemotaxis of various substances, because it is possible to make an exact quantitative determination of the leucocytes that have migrated from the blood clot and adhere to the surfaces containing the tested substance.

2. The calcium ion is the only inorganic ion *per se* which is found to be positively chemotactic under the conditions of these experiments. It is markedly chemotactic in all concentrations and in all combinations, except the citrate. Here the negative chemotaxis of the citrate ion neutralizes the positive chemotaxis of the calcium ion, and neutrality of chemotactic effect results.

3. The sodium and magnesium ions themselves are neutral. Magnesium and sodium salts are dependent upon the negative ion with which the magnesium or sodium is combined for such positive or negative chemotaxis as is exhibited. All the phosphates of sodium, whether tri-, di-, or monobasic salts, are markedly positively chemotactic, and when combined with other reagents which are themselves neutral or negatively chemotactic, produce marked positive chemotaxis. The blood of a person who has taken phosphates either by mouth or intravenously shows a great increase in chemotaxis with sodium phosphate, with calcium chloride, and even with sodium chloride which is ordinarily neutral.

4. All potassium salts are negatively chemotactic.

5. Many substances act synergistically as regards chemotaxis; *e.g.*, when strontium and magnesium salts are mixed there is a marked increase in chemotaxis. Sodium phosphate acts synergistically with calcium chloride.

6. Mercury salts fix the leucocytes in this method so that their influence on chemotaxis cannot be determined.

7. Morphine and morphine salts are positively chemotactic; this is contrary to the results obtained by others with different methods.

8. Substances which produce a very acute inflammation, such as cantharidin, histamine, or turpentine, are found to be positively chemotactic by this method, but substances, such as mustard gas, which produce a marked necrotizing effect are found to be negatively chemotactic, or neutral, though physiologically they would appear to be positively chemotactic.

9. All amino-acids and amines are positively chemotactic to a certain extent. It seems that the longer the carbon chain, the greater the degree of chemotaxis, though this is not absolute. Tyramine is one exception to this, for it causes a peculiar clumping of the cells, so that it is impossible to count the number adhering, and thus determine whether or not tyramine is positively chemotactic.

10. The time that the blood of animals is examined after eating makes a marked difference in the number of cells adhering, for shortly after eating, within 30 minutes, very many more cells will adhere to the agar than at a later time.

11. The blood of different species of animals reacts differently towards different reagents. The chemical composition of these agents seems to have nothing to do with this difference in reaction as far as we could determine.

12. With frozen serial sections it has been found that the depth of penetration of the leucocytes into the agar is proportional to the positive chemotaxis produced by the substance combined with the agar, as demonstrated by the number of leucocytes adherent to the walls of the test chambers.

WANDERING CELLS, ENDOTHELIAL CELLS, AND FIBROBLASTS IN CULTURES FROM HUMAN LYMPH NODES.

By WARREN H. LEWIS, M.D., and LESLIE T. WEBSTER, M.D.

(*From the Department of Embryology of Carnegie Institution of Washington and the Department of Pathology of Johns Hopkins Medical School, Baltimore.*)

Plates 26 to 32.

(Received for publication, April 22, 1921.)

In recent communications on the cultivation of human lymph nodes, lymphocytes and giant cells have been described.[1, 2] This paper, to complete the series, will be concerned with observations on wandering cells, reticulum cells, endothelial cells, and fibroblasts, with a discussion as to the significance of their morphological and physiological similarity.

Lymph glands, excised aseptically from patients in the Johns Hopkins Hospital, were brought to the laboratory and planted from ½ to 2 hours after their removal. They were cut into small bits in sterile Locke-Lewis solution, placed as usual upon a sterile No. 1 cover-slip, covered with a drop of homogenous or autogenous plasma, inverted over a hollow ground slide rimmed with vaseline, and immediately put into an incubator with a temperature of 37.5°C. Twenty to sixty cultures were prepared from each gland; this afforded an opportunity for a considerable number of studies lasting from 2 hours to 3 weeks.

The seven cases of normal glands showed relatively few of the cells under discussion, as did the cases of melanosarcoma, carcinoma, and lymphoid and myeloid leucemia. But in the two cases of sarcoma, the three cases of chronic lymphadenitis, two cases of Hodgkin's disease, and seven cases of tuberculous lymphadenitis, they were very

[1] Lewis, W. H., and Webster, L. T., *J. Exp. Med.*, 1921, xxxiii, 261.
[2] Lewis, W. H., and Webster, L. T., *J. Exp. Med.*, 1921, xxxiii, 349.

numerous, and in each case the relative and absolute quantity of cells seen in the cultures was proportional to that seen in the corresponding histological sections stained with hematoxylin and eosin.

Wandering Cells.

Histological sections of normal and pathological human lymph glands show deeply staining mononuclear cells with abundant cytoplasm, scattered about the sinuses and through the lymph tissue. To this most common type of loose tissue cell, found as well in chronic inflammatory areas and foci of cellular infiltration, pathologists give the name wandering cell. Its origin will be referred to later.

After 2 or 3 hours incubation, wandering cells may be seen coming from cultures of normal glands, tuberculous glands, and glands with Hodgkin's disease and metastatic tumor. They follow the migrating lymphocytes which are first to leave the tissue fragment and form a ring about the explant between the cover-slip and plasma clot. They are irregular in size and shape but when flattened out on the cover-slip, they measure about 0.024 by 0.016 mm. They consist of a delicate clear zone of ectoplasm and an inner, slightly more opaque zone of endoplasm which contains mitochondria, fat globules, neutral red granules and vacuoles, and a single nucleus (Figs. 1 to 8 and 30).

The fat globules appear greenish and refractile; they take a Sharlach R stain readily. Although they vary in number, size, and distribution in cells at different ages, during the early hours of observation they are generally few in number, regular in size, and scattered indiscriminately throughout the endoplasm.

A few neutral red granules and an occasional vacuole are seen, but no note concerning a definite grouping of granules about a centrosphere area was made at this time.

The nuclear material has a waxy appearance and occupies from one-third to one-half of the cell volume. With the cell spread out at rest, the nucleus tends toward an oval or bean shape, but when the cell is actively moving, it adapts itself to conditions determining movement by assuming all possible forms from bizarre to spindle and dumb-bell outlines.

The cells move rapidly and generally away from the explant, although they frequently change their direction or remain motionless for a considerable period of time. The clear ectoplasm, which seems to be fluid, pushes out in a fan-shaped manner, advancing and retracting its pseudopods. Into this protrusion a few fat globules with active Brownian movement are seen to flow, followed by the nucleus. Finally the posterior part of the cell draws up and the crawling, flowing motion may temporarily cease. The average rate of migration is roughly estimated at 0.0306 mm. per minute. This is considerably faster than the migratory rate of lymphocytes (Fig. 14).

At 24 hours there is moderate migration of wandering cells in cultures from normal glands and glands with carcinoma metastases, while in cultures from chronic lymphadenitis, tuberculous lymphadenitis, and glands with sarcoma metastases and Hodgkin's disease, the wandering cells are present in great numbers. They are scattered radially about the explant and are most numerous in its vicinity.

Usually the cells show no alteration. The ectoplasm, still clear, contains rod-like and granular mitochondria, conspicuous when stained with Janus black No. 2. In the more inactive cells the ectoplasm tends to become irregular.

In the endoplasm fat globules and vacuoles are larger and more numerous. There are many neutral red granules, differing in size and shape. They may be scattered indefinitely throughout the cell or grouped at one side of the nucleus radially about a central clear area. Most of the cells are still active; nucleus and cytoplasm change shape and position rapidly.

These cells are active phagocytes. In the infected cultures, bacteria are crowded about the cell membrane and are contained in large numbers in the endoplasm. In other cultures hemoglobin pigment and disintegrating lymphocytes are engulfed by the wandering cells.

A few cells are greatly altered in appearance. They are much smaller and appear dark and shrivelled. The contracted ectoplasm shows many hair-like processes clinging to the cover-slip. The endoplasm appears as a shrunken, irregular, circular mass suspended from the under surface of the glass by the ectoplasmal processes. It

often contains one or two very large fat globules or vacuoles taking the neutral red stain. When visible, the nucleus is usually dark, sausage-shaped, and pushed to one side of the cell (Figs. 9 to 13).

At 48 hours an occasional wandering cell is seen spread out upon the cover-slip. The fat globules are larger and the neutral red area is composed of a rosette arrangement of granules at one side of the nucleus. Instead of active migration, movement is limited to changes in shape of the cell and relations of its component parts. By far the greater number are shrivelled and inactive, clinging to the cover-slip by spidery ectoplasmal processes.

At 5 days no active wandering cells are seen.

In brief, then, the wandering cell, found in the sinuses of lymph glands, about chronic inflammatory areas, and associated with so called round cell infiltrations, migrates from cultures of human lymph nodes. It appears in 2 or 3 hours, shortly after the lymphocytes, continues to move for about 48 hours, then gradually becomes quiescent, shrivels, and dies. Its structure and physiological characteristics make it easily recognizable.

Endothelial Cells.

In describing the histology of lymph nodes, text-books of Stöhr,[3] Shäfer,[4] and Szymonowicz[5] refer to lymphatic endothelium as lining capsule, trabeculæ, lymph sinuses, and continuing as branched cells to form the general supporting framework, or reticulum. However, in our observations on migrating cells, we were unable to differentiate blood vessel endothelium, lymphatic endothelium, and reticular endothelium so the term endothelial cell will be used to designate a group of cells seen in cultures of normal and pathological lymph nodes morphologically and physiologically similar to each other and

[3] Stöhr, P., in Schultze, O., Lehrbuch der Histologie und der mikroskopischen Anatomie des Menschen mit Einschluss der mikroskopischen Technik, Jena, 16th edition, 1915, 141.

[4] Schäfer, E. A., The essentials of histology, Philadelphia and New York, 10th edition, 1916, 231.

[5] Szymonowicz, L., Lehrbuch der Histologie und der mikroskopischen Anatomie mit besonderer Berücksichtigung des menschlichen Körpers einschliesslich der mikroskopischen Technik, Würzburg, 3rd edition, 1915, 164.

to those seen in fixed preparations answering the above description. Endothelial cells migrate from explants of normal and pathological glands after 24 to 48 hours incubation. They are not active, so unless one chances to see a sudden streaming of granules and nucleus into advancing ectoplasm as the cell lengthens and momentarily changes its position, or unless by prolonged observation a rough average rate of 0.0002 mm. per minute is determined, the cells appear motionless. A predilection for clear areas between media or explant and cover-slip gives opportunity for careful study over considerable periods of time.

The typical endothelial cell, almost circular, measures about 0.04 mm. in diameter. One can distinguish clear ectoplasm with mitochondria and endoplasm containing nucleus, fat globules, mitochondria, and neutral red granules and vacuoles. The ectoplasm, when seen in its most characteristic form, appears as a broad circular band about three-fifths of the cell area whose outline, with irregular projections and indentations, changes somewhat from hour to hour. Scattered through the otherwise homogeneous ectoplasm are mitochondria in short threads or granules. Fat globules, limited to the endoplasm, vary in number, size, and distribution. Usually they are small and regularly scattered about the nucleus and centrosphere area. The neutral red granules form a rosette at one side of the nucleus. Small near the central clear area, they increase in size toward the periphery. About the neutral red area are mitochondria in short threads and granules. The nucleus is large, oval or bean-shaped, waxy in appearance, and motionless (Figs. 15 to 17).

Besides the so called characteristic forms, there are many variable cells tending on the one hand toward the smaller wandering cells and on the other hand toward the spindle or fibroblast cells. As these intermediate forms are studied, transitions may be seen, forming an unbroken series from wandering cells to endothelial cells and from endothelial cells to giant or spindle cells.

The transitions from endothelial cells to wandering cells are characterized by a decrease in the size of the cell and marked irregularity of outline. The circular shape gives way to irregular polyhedral and bizarre forms with ectoplasm seen chiefly as projections, hair-like threads, or long processes. Fat globules and neutral red granules

and vacuoles are more compact in the dense endoplasm, and the nucleus is smaller and less distinct. When these intermediate forms are actively ameboid or when they contain large numbers of disintegrating lymphocytes (Figs. 32 and 33) the cytological picture is, of course, modified, but in general the changes are as described above.

The transition forms from endothelial cells to spindle cells are more striking. The tendency to assume the bipolar shape is accompanied by an increase in surface area. Ectoplasm, very prominent, may flow out at one end and develop a long tail process at the opposite end while endoplasm and contents appear unchanged. Other cells show a triangularly shaped ectoplasm, some a dumb-bell elongation, and still others an outline resembling typical fibroblasts. No changes are observed in endoplasm or its constituent parts; the alteration is chiefly that of outline (Figs. 18 to 23, 28, and 29).

In the communication on giant cells[2] note was made of transition forms from large wandering cells or endothelial cells to giant cells with two, three, and four or more nuclei (Figs. 24 to 26).

12 to 24 hour observations on single endothelial cells determined their slow rate of motion and the infrequency of amitotic nuclear division and also brought to light actual changes in shape of the cells. Shrinking was observed with developing spicules and blebs; sudden acceleration of motion with a bipolar lengthening of the cell and a rapid flowing of nucleus and granules was not uncommon. The cell would move a short distance and then reassume its rounded form.

After 3 or 4 days incubation many cultures show a predominance of large cells of the endothelial type whose ectoplasm is either spread out irregularly upon the cover-slip or is branching and connected with adjacent cells. Here, all transitions from endothelial cells to fibroblasts may be seen; many branching cells contain two or three nuclei; typical giant cells are frequent (Fig. 31). It is possible that these may be true reticulum cells (Fig. 27).

At the end of the 1st week, lymphocytes and wandering cells have practically disappeared, leaving endothelial cells, giant cells, and transitional cells scattered through a network of fibroblasts (Fig. 34). Some of the endothelial cells shrivel, others disintegrate, so that after 16 days, very few remain.

In brief, then, endothelial cells migrate from explants of normal and pathological lymph glands after 24 to 48 hours incubation. They are large, almost without motion, constant in their cytological characteristics but are accompanied by related forms which simulate wandering cells or fibroblasts. The former may be active or phagocytic, the latter may show branched processes connecting similar cells.

Fibroblasts.

Fibroblasts occur in the capsules and trabeculæ of normal lymph glands and in scar tissue of many pathological glands. They appear in cultures from normal and pathological lymph nodes after 48 to 72 hours incubation. At this time the cells are usually spindle-shaped and migrate between cover-slip and plasma with their long axes parallel to radii from the explant. After the 4th or 5th day they may be branched and form a loose reticulum or network about the tissue fragment. They are often seen at this time in cultures which are otherwise quite devoid of growth.

Cytologically, they are similar to the fibroblasts of chick embryos described by Lewis.[6] The ectoplasm is usually bipolar and clear except for a few thread-like or granular mitochondria. Fat globules vary in number, size, and distribution. Neutral red granules and vacuoles may be grouped about a definite centrosphere area or may be scattered through the endoplasm. The nucleus is waxy in appearance and oval or bean-shaped (Fig. 35).

After 18 or 20 days, fibroblasts are still present and vigorous in many cultures.

DISCUSSION.

In spite of Maximoff's recent work describing transition forms between lymphocytes and reticulum cells,[7] we have been unable to demonstrate any such relationship. However, we are impressed with the similarity of wandering cells to endothelial cells. Morphologically, differences are of apparent size and outline while the quality of nucleus, endoplasm and contents, and ectoplasm is the

[6] Lewis, W. H., *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.

[7] Maximoff, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 222.

same. Physiological variations concern degree rather than nature of activity. Prolonged observations of single endothelial cells demonstrate short periods of activity accompanied by changes in shape. The frequent series of transitional forms lend further support to the theory of Marchand[8] that the wandering cells of lymph glands arise from the endothelial cells.

The large branching cells, appearing after the 3rd or 4th day, described with the endothelial cells, may well be true reticulum cells.

CONCLUSIONS.

Wandering cells migrate from cultures of human lymph nodes after 2 or 3 hours incubation; they are actively ameboid and phagocytic.

Endothelial cells appear in the plasma clot after 24 to 48 hours incubation. They are relatively inactive and less phagocytic.

Fibroblasts are seen after 48 hours incubation. They are inactive and not phagocytic.

The probable origin of wandering cells from endothelial cells is discussed.

EXPLANATION OF PLATES.

PLATE 26.

FIGS. 1 to 13. Wandering cells from cases of tuberculous lymphadenitis (Series 18). Fixed after 21 hours incubation. × 750.

PLATE 27.

FIG. 14. Normal gland (Series 13). 4 hours incubation at 37.5°C. A wandering cell drawn at 1 minute intervals. Arrow points to explant. × 559.

PLATE 28.

FIGS. 15 to 17. Typical endothelial cells from cases of tuberculous lymphadenitis (Series 18–9). Fixed after 2 days incubation. × 750.

FIGS. 18 to 23. Transitional forms from endothelial cells to spindle cells from cases of sarcoma and tuberculous lymphadenitis (Series 8–9). 3 and 5 day cultures. × 750.

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[8] Marchand, F., *Verhandl. deutsch. path. Ges.*, 1913, xvi, 5.

PLATE 29.

FIGS. 24 to 27. Giant cells from a case of tuberculous lymphadenitis (Series 9). 5 day culture. × 750.

FIGS. 28 and 29. Branching transitional endothelial cells from a case of tuberculous lymphadenitis (Series 9). 5 day culture. ×750.

PLATE 30.

FIG. 30. Wandering cells from a case of tuberculous lymphadenitis (Series 9). Fixed after 21 hours incubation.

FIG. 31. Endothelial cells and giant cells from a case of tuberculous lymphadenitis (Series 9). Fixed after 4 days incubation.

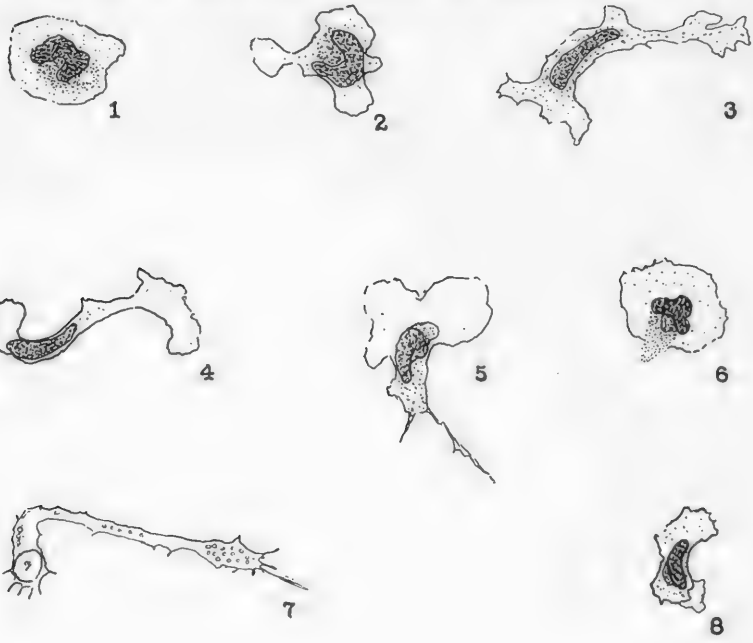
PLATE 31.

FIGS. 32 and 33. Phagocytosis by wandering cells and endothelial cells. Fig. 32. Tuberculous lymphadenitis (Series 7). 4 day culture. Fig. 33. Tuberculous lymphadenitis (Series 18). Fixed after 2 days incubation.

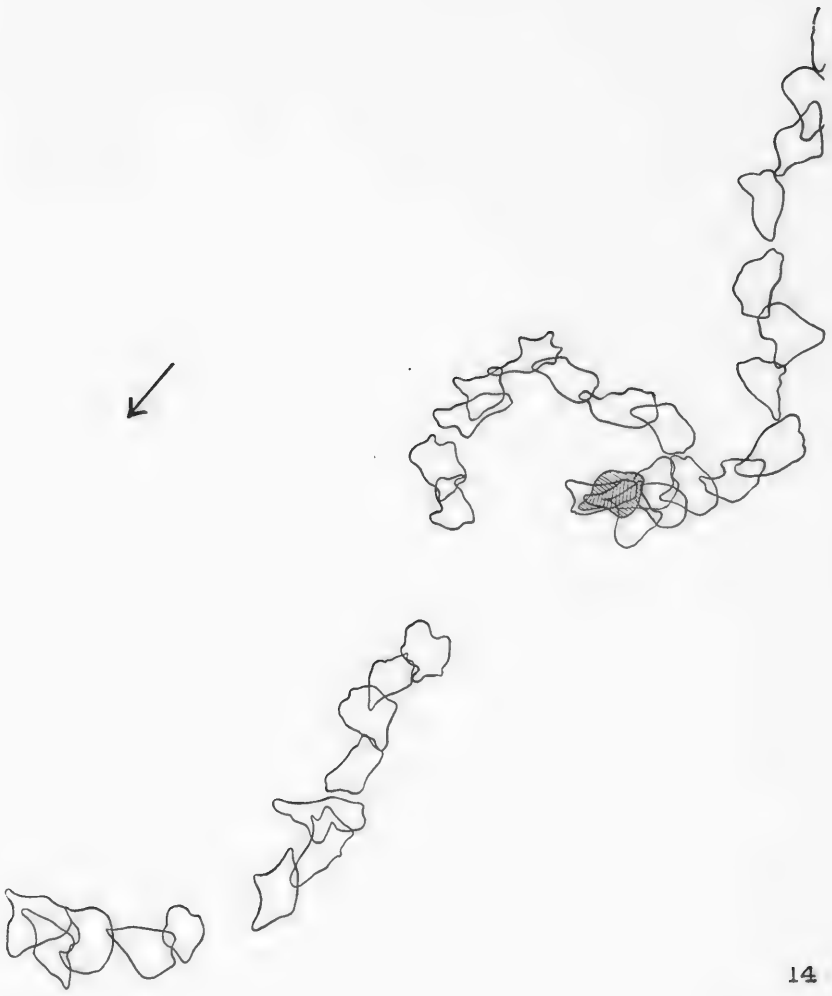
PLATE 32.

FIG. 34. Endothelial cells, transitional cells, and spindle cells from a case of tuberculous lymphadenitis (Series 18). 7 day culture. Fixed.

FIG. 35. Spindle cells from a case of tuberculous lymphadenitis (Series 9). 9 day culture. Fixed.



(Lewis and Webster: Cultures from human lymph nodes.)



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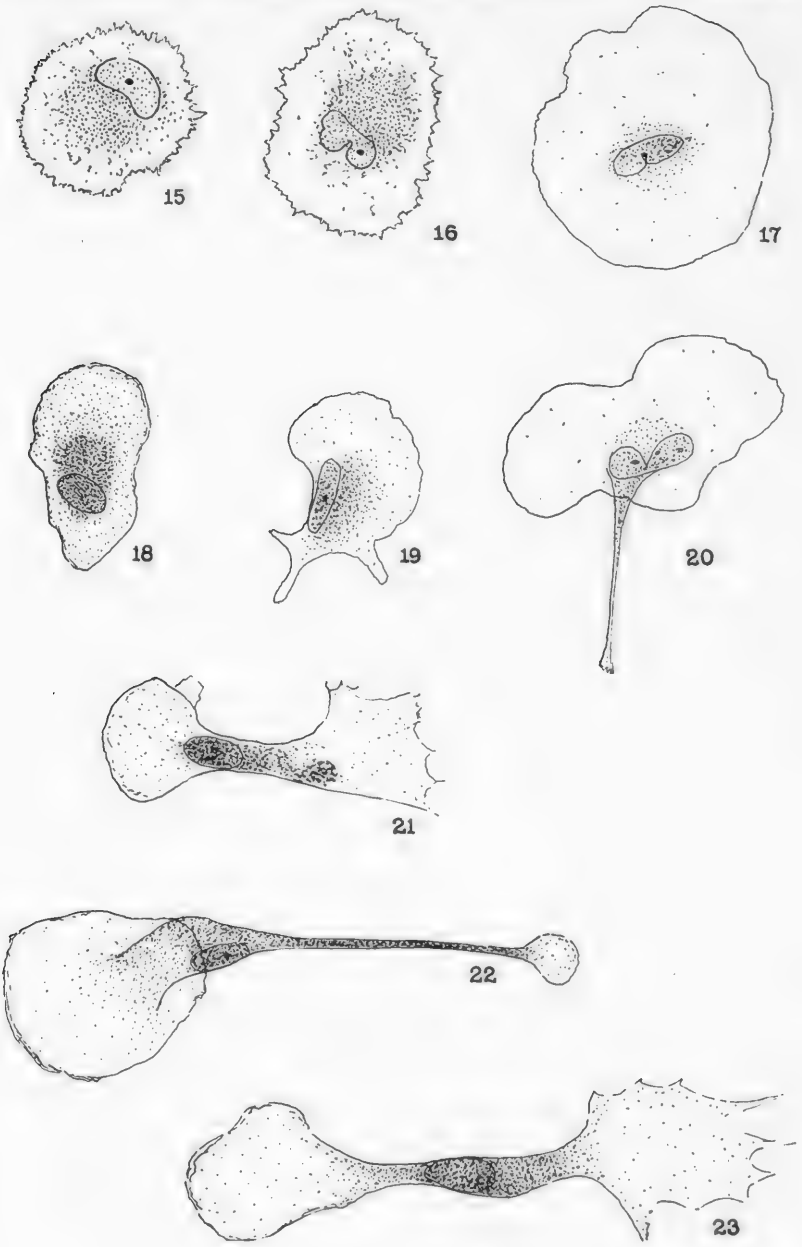

(Lewis and Webster: Cultures from human lymph nodes.)

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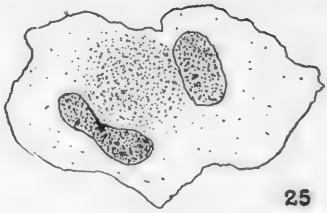

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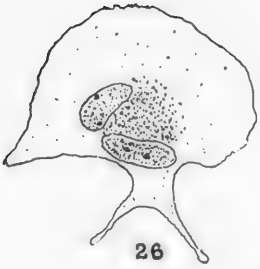

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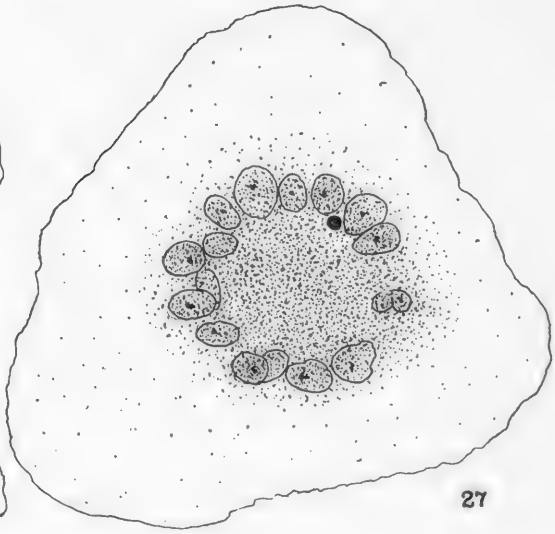

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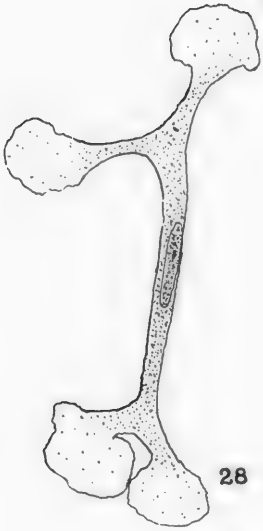

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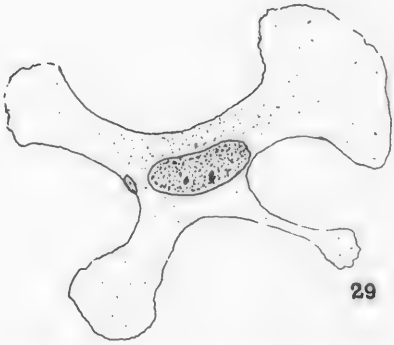

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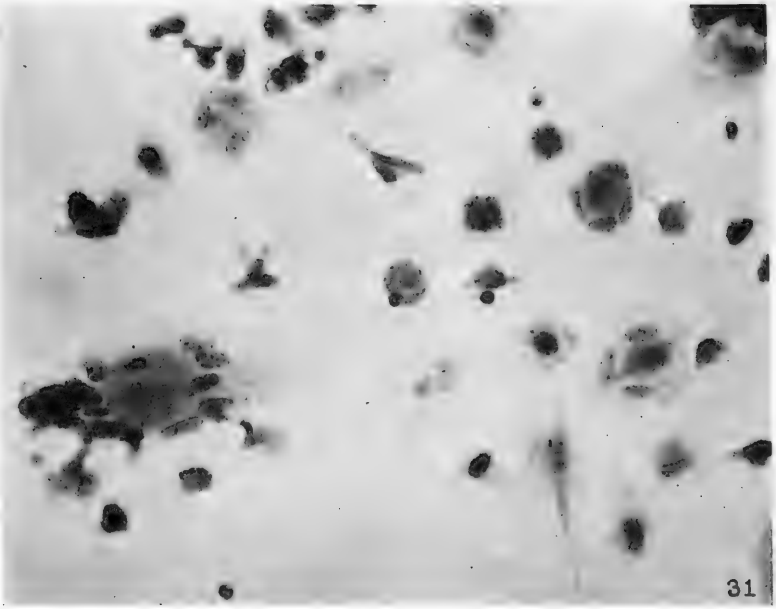
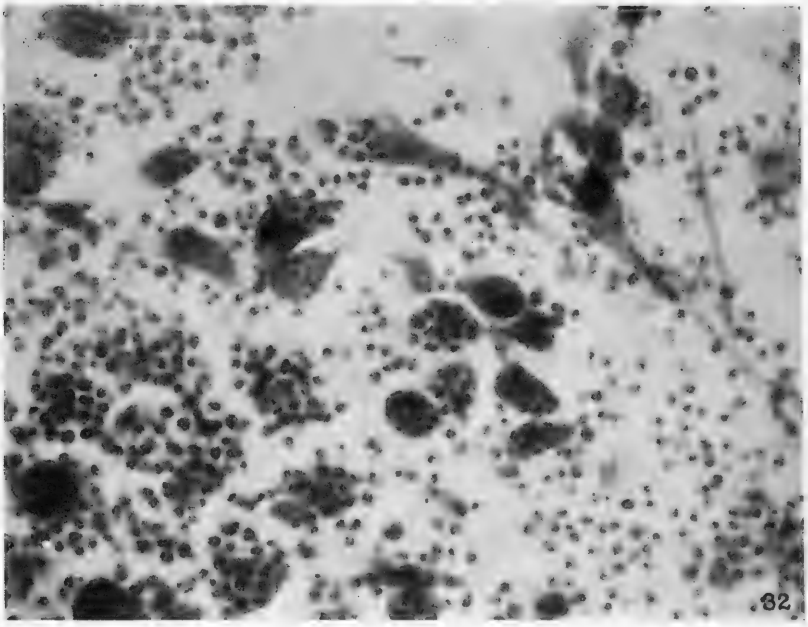
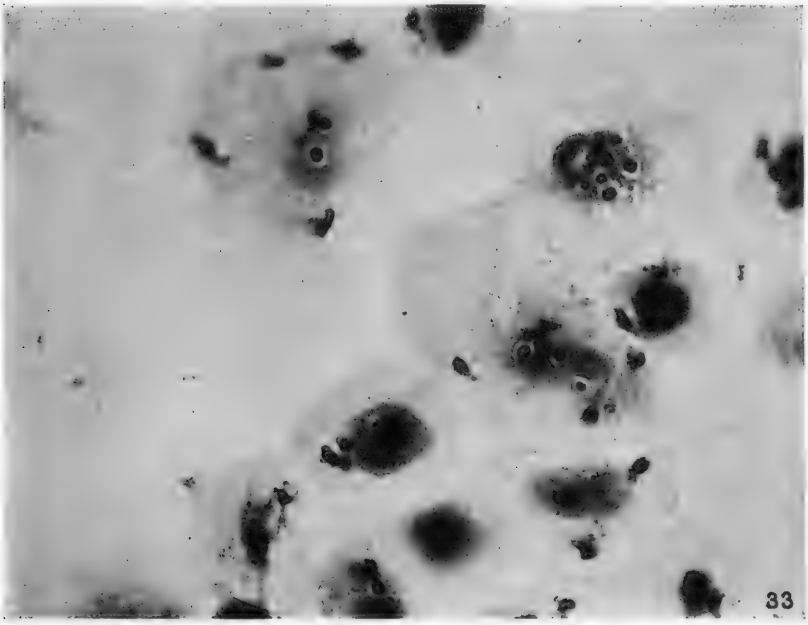
(Lewis and Webster: Cultures from human lymph nodes.)

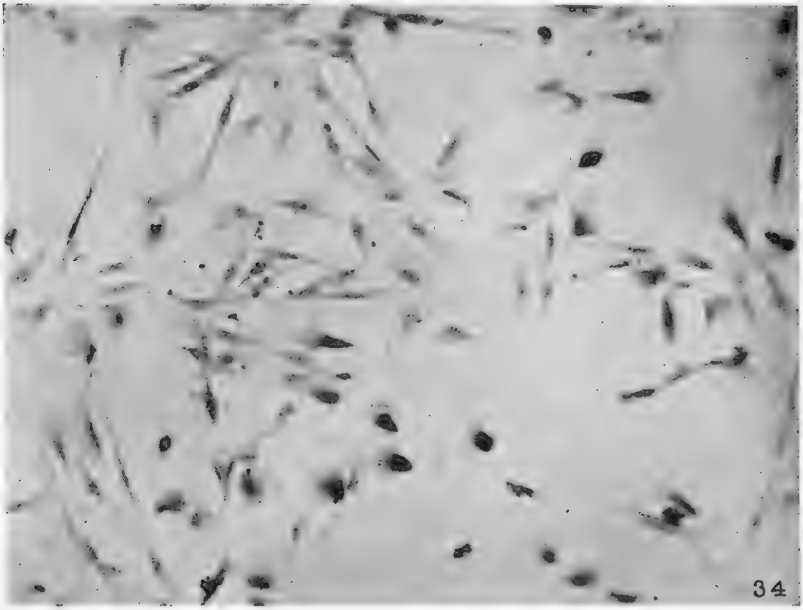


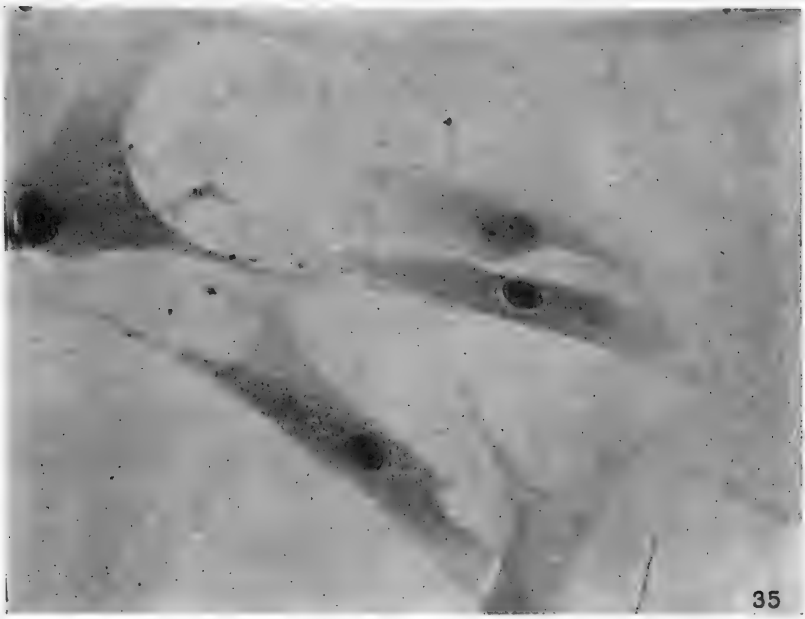
(Lewis and Webster: Cultures from human lymph nodes.)





(Lewis and Webster: Cultures from human lymph nodes.)





(Lewis and Webster: Cultures from human lymph nodes.)

THE INFLUENCE OF THYROID PRODUCTS ON THE PRODUCTION OF MYOCARDIAL NECROSIS.

By ERNEST W. GOODPASTURE, M.D.

(*From the Department of Pathology of Harvard Medical School, Boston.*)

PLATE 33.

(Received for publication, May 11, 1921.)

In a recent study of hearts from cases of hyperthyroidism dying of myocardial exhaustion, the writer found acute necrosis of cardiac muscle, in one instance so diffuse as to involve a large part of the left ventricular wall.[1] The character of necrosis was that usually associated with extreme intoxication by acute infections such as diphtheria or scarlet fever, and more commonly occurring in youth. Since there was no evidence of such a severe intoxication in these cases it seemed probable that the myocardium was in a condition which rendered it much more easily injured than usual.

Inasmuch as practically every case of hyperthyroidism at one time or another shows cardiac symptoms, which not infrequently progress in severity until the patient becomes permanently incapacitated by myocardial exhaustion, it is generally considered that products of the diseased thyroid gland are the immediate cause of injury to the heart, though the manner in which their action is exerted is not understood.

The presence of destructive lesions of the myocardium in cases of exophthalmic goiter has been demonstrated by Fahr,[2] who attributes the injury to direct action of products of the diseased thyroid gland upon heart muscle fibers.

In the cases of hyperthyroidism studied by the writer there was no indication of an infection of sufficient virulence to be alone responsible for the necrosis. It was difficult also to think that so acute and uniform a lesion was the outcome of a slowly progressive degeneration

[1] Goodpasture, E. W., *J. Am. Med. Assn.*, 1921, lxxvi, 1545.

[2] Fahr, Th., *Centr. allg. Path. u. path. Anat.*, 1916, xxvii, 1.

of the myocardium resulting from continued action of products of a diseased thyroid, although such an explanation might suffice to account for the general disease of the heart which had persisted over a considerable period of time.

It seemed reasonable to suppose that these hearts overstimulated by disease of the thyroid and laboring in a condition bordering on exhaustion, were in a state of greater susceptibility to injury by toxic substances such as may have resulted from a relatively mild terminal infection which under other circumstances would not have materially injured the myocardium.

The following study was undertaken to determine, first, what demonstrable effect feeding desiccated thyroid gland, or intravenous administration of crystalline thyroxin would produce in the myocardium; then, whether the effect of these substances would cause the heart to be more readily injured by toxic agents, notably chloroform.

Material.

Rabbits free of infection were used for the experiments. It was found most satisfactory to use young adult males weighing from 2,000 to 2,500 gm. Unless animals of approximately the same age, weight, and condition were used there was a great deal of variation in the reaction of different individuals to the same dosage of thyroid products. These substances are more toxic for young rabbits than for old ones, and for rabbits of the same age and weight they seem less toxic during pregnancy, though the fetuses are readily killed.

In the first set of experiments two samples of desiccated thyroid gland containing 0.2 per cent iodine were used. The desired amount was mixed with water, and the thin paste formed was administered orally by means of a medicine dropper inserted far back in the mouth.

Crystalline thyroxin (Squibb's) containing about 65 per cent iodine was likewise used but was given by intravenous injection. 10 mg. were dissolved in 5 cc. of distilled water to which 2 drops of 10 per cent sodium hydroxide had been added. The solution was then sterilized in boiling water and used up within 2 or 3 days. In preparations kept longer, not infrequently a granular precipitate appeared, but until this happened there did not seem to be any diminution in the effectiveness of the solution.

In the second set of experiments rabbits treated with dried thyroid or crystalline thyroxin received chloroform by inhalation in addition. Chloroform (U.S.P.) for anesthesia was used for this purpose.

Method.

Rabbits were kept in the laboratory several days on a diet of oats, hay, and carrots or spinach until their weight became constant or began to increase. Daily weight and pulse rate were recorded, and symptoms noted throughout the experiment. The first set, *i.e.* those receiving dessicated thyroid, received 1 gm. daily, suspended in water, administered through a medicine dropper. Those receiving thyroxin were given every 2nd or 3rd day 1 mg. dissolved in 0.5 cc. of alkaline solution and injected into an ear vein.

The pulse was counted by means of a stethoscope applied to the chest while the animal was quiet, and in some instances electrocardiographic tracings were made. As the rate sometimes rose to 400 or more per minute, it was often necessary to employ the method of dotting paper with a pencil, thus recording each impulse for 30 seconds to 1 minute and counting them afterward, a procedure shown by Levine to be very accurate.[3] The animals soon become accustomed to being handled and very uniform results were obtained. The animals in the first set, which received desiccated thyroid gland or thyroxin alone, were treated for from 2 to 3 weeks, and except in an occasional instance they were killed by a blow on the head and the tissues were fixed immediately. The average normal pulse rate for rabbits is around 200 per minute, though individuals show considerable variation. In the second group of experiments when the pulse rate rose to about 300 per minute the rabbits were given chloroform by inhalation for 1 hour and were killed by a blow on the head 24 to 72 hours later. Chloroform was given by the drop method or by holding the open mouth of the bottle above the nose just far enough removed to prevent spasmodic inhibition of respiration. The animals were never deeply anesthetized, the corneal reflex was not lost, and after the anesthetic was removed they immediately got on their feet.

[3] Levine, S. A., *J. Am. Med. Assn.*, 1920, lxxv, 795.

Tissues were fixed in Zenker's solution and stained by the eosin-methylene blue method for routine microscopic examination, and in other fixatives for special studies.

The Effect of Desiccated Thyroid and Thyroxin.

While it is appreciated that neither desiccated thyroid nor thyroxin may contain or represent the real secretion product of the thyroid gland in health or disease,[4] they both induce physiological activities more or less analogous to those present in patients with hyperthyroidism; namely, tachycardia, increased metabolism, nervousness, etc. It is estimated regarding dosage that 1 gm. of desiccated thyroid gland containing 0.2 per cent iodine under favorable conditions of preparation will contain approximately the equivalent of 1 mg. of thyroxin. I have found, however, with the preparations used in rabbits that 1 mg. of thyroxin injected intravenously is considerably more potent in increasing the pulse rate and inducing loss of weight than 1 gm. of desiccated thyroid administered by mouth.

There is a great deal of variation in the way different rabbits react to the same dosage. Old individuals are very resistant to the quantities which were employed; younger ones vary in susceptibility according to age, weight, and general condition. The more refractory animals gradually acquire a tolerance to large doses, while the susceptible ones rapidly emaciate and die, apparently from thyroxin intoxication. They show toward the end extreme muscular weakness, being unable to hold up their heads. Diarrhea is a frequent early symptom and may persist, but often disappears on continued treatment. As was noted by Wilson and Kendall,[5] an animal will sometimes recover from a serious degree of intoxication even though it continues to receive large doses of thyroxin. The rabbits eat very little for the first few days and those most intoxicated abstain from food almost entirely, which explains largely the rapid loss in weight. As treatment continues, frequently the hair becomes dry,

[4] The delayed action of these substances does not harmonize with the rapid effects produced by Cannon on stimulation of the thyroid, and attributable to functional activity of this gland.

[5] Kendall, E. C., *Tr. Assn. Am. Phys.*, 1915, xxx, 420. Wilson, L. B., and Kendall, E. C., *Tr. Assn. Am. Phys.*, 1915, xxx, 458.

lusterless, and tends to fall out. Excitability and restlessness increase. No sugar has been found in the urine.

The two effects which I have followed most carefully are those on pulse rate and body weight. The more constant of the two is an increased heart rate which practically always occurs to a greater or less degree with or without loss in weight. There is no constant relation between the two. The cardiac rhythm has always remained regular. Under the influence of excitement or exercise the rate rapidly increases, sometimes 25 per cent, but subsides within a few minutes to a constant level.

The rise in pulse rate begins 24 to 48, sometimes 72 hours after the initial dose, somewhat sooner after thyroxin than after desiccated thyroid. It rapidly increases to 50 or 75 per cent above the normal rate, and maintains a high level under treatment. The pulse becomes more forceful and the ear veins appear to dilate and contract more readily than normally. No constant elevation of temperature has been noted.

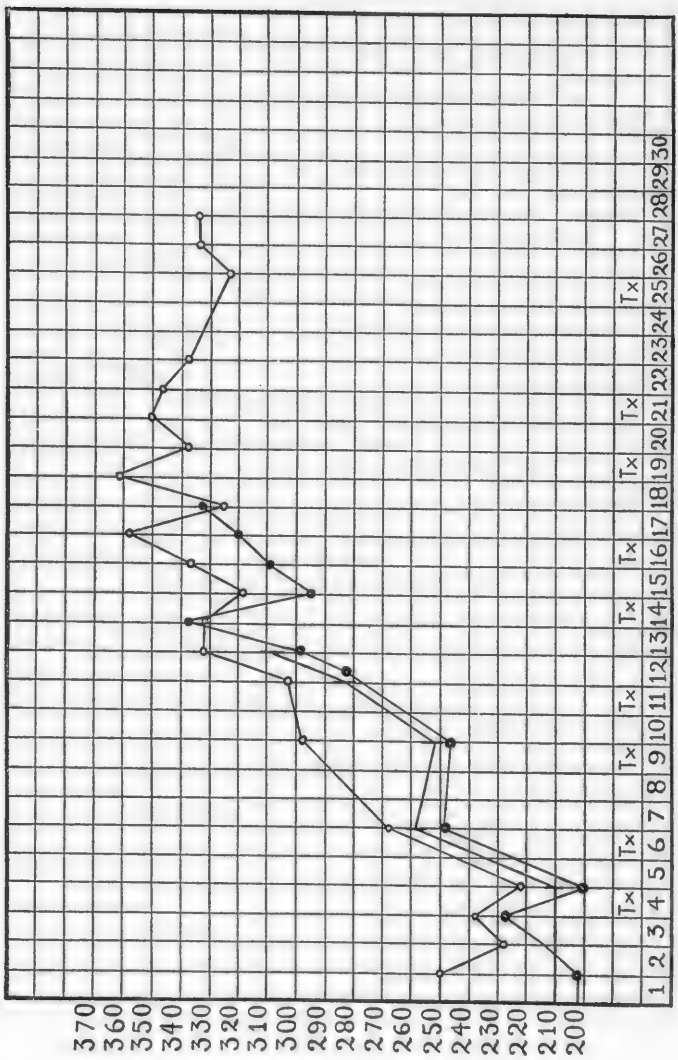
A progressive secondary anemia accompanies the severer degrees of intoxication.

Text-figs. 1 and 2 illustrate the relative increase in pulse rate and loss of weight in the same group of animals after thyroxin administration.

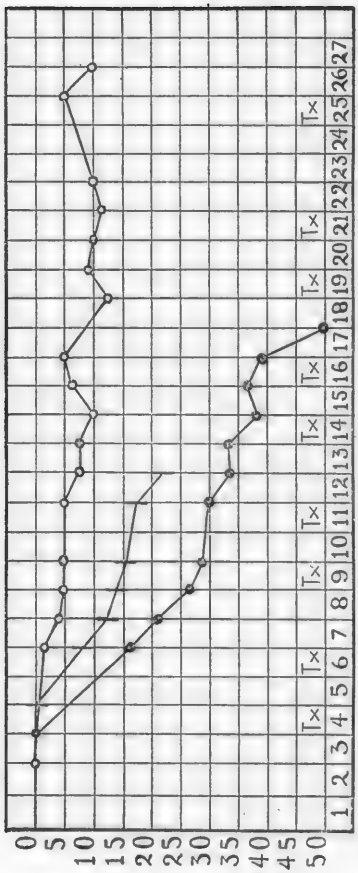
To observe the changes produced in the myocardium by feeding thyroid gland the hearts of seven rabbits were studied. Two had received 1 gm. of thyroid daily for 15, two 16, one 17, one 19, and one 26 days. A typical protocol follows:

Rabbit 1.—Brown; young adult male.

Aug. 19, 1920.	Weight 2,322 gm.;	pulse 170.					
" 20.	" 2,361 "	" 154.					
" 21.	" 2,380 "	" 156.					
" 23.	" 2,446 "				thyroid	1	gm.
" 24.	" 2,375 "	pulse 176;	"	1	"		
" 25.	" 2,267 "	" 188;	"	1	"		
" 26.	" 2,206 "	" 204;	"	1	"		
" 27.	" 2,132 "	" 206;	"	1	"		
" 28.		" 256;	"	1	"		
" 30.	Weight 1,997 gm.;	" 244;	"	1	"		
" 31.	" 1,970 "	" 272;	"	1	"		



TEXT-FIG. 1. Increase in pulse rate following the intravenous injection of crystalline thyroxin. The abscissæ indicate days, the ordinates pulse rate. *Tx*, received 1 mg. of thyroxin crystals. |—|—| Average increase in five rabbits. ○—○—○ Increase in Experiment 2. ●—●—● Increase in Experiment 4.



TEXT-FIG. 2. Percentage loss in weight following the intravenous injection of crystalline thyroxin. *Tx*, received 1 mg. of thyroxin. The abscissæ indicate days, the ordinates percentage loss in body weight. |—|—| Average per cent loss in body weight. ○—○—○ Per cent loss in Experiment 2. ●—●—● Per cent loss in Experiment 4. The same group as in Text-fig. 1.

Sept. 1, 1920. Weight 1,933 gm.; pulse 250; thyroid 1 gm.
" 2. " 1,922 " " 262; " 1 "
" 3. " 1,885 " " 308; " 1 "
" 4. " 1,868 " " 1 "
" 7. " 1,911 " pulse 268; " 1 "
" 8. " 1,905 " " 1 "
" 9. " 1,878 " " 1 "
" 10. " 1,877 " " 1 "
" 11. " 1,852 " " 1 "
" 13. " 1,928 " " 1 "
" 15. pulse 312; " 1 "
" 17. Killed. Tissues fixed in Zenker's solution. Lungs normal. Heart shows no gross abnormality. Other organs show no gross changes.

The hearts from a similar group of rabbits that had received thyroxin intravenously over periods of 9, 14, 18, 20, and 23 days respectively were studied in the same way. The following protocol is an instance of extreme intoxication, the animal being killed in an almost moribund condition.

Rabbit 2.—White; young adult male.
Oct. 7, 1920. Weight 2,045 gm.; pulse 200.
" 8. " 2,180 " " 226.
" 9. " 2,141 " " 232.
" 11. " 2,222 " " 200; thyroxin 1 mg.
" 13. " 2,010 " " 240; " 1 "
" 14. " 1,980 "
" 15. " 1,940 "
" 16. " 1,985 " pulse 212; thyroxin 1 mg.
" 18. " 1,867 " " 244; " 1 "
" 19. " 1,750 " " 276; " 1 "
" 20. " 1,665 " " 312; " 1 "
" 21. " 1,603 " " 292; " 1 "
" 22. " 1,595 " " 272; " 1 "
" 23. " 1,525 " " 300; " 1 "
" 24. " 272; " 1 "
" 25. Weight 1,405 gm.; " 268; " 1 "
" 26. " 1,468 " " 220; " 1 "
" 27. " 1,422 " " 252; " 1 "
" 28. " 1,330 " " 240; " 1 "
" 29. " 1,221 " " 256; " 1 "
" 30. Very weak, especially muscles of neck. Head bends over and animal cannot raise it. Greatly emaciated. Killed.

Autopsy.—Lungs clear. Heart weight 4.75 gm. No gross changes. Other organs small and atrophic. Stomach almost empty. Brownish blood streaks and small erosions of mucosa with edema of wall in places. Adrenals smaller than normal, pink and meaty, with no obvious cortical fat. Spleen small and brownish. Tissues fixed in Zenker's solution.

Four of the five rabbits which received similar doses of thyroxin were intoxicated in the above manner. One was relatively resistant and although it showed a marked increase in pulse rate, there was little loss in weight.

None of the hearts from these two groups of animals, *i.e.* the one receiving desiccated thyroid, the other crystalline thyroxin, showed any gross lesion excepting atrophy in the case of those animals which rapidly emaciated while receiving thyroxin. Numerous sections were cut from both ventricles and auricles. Microscopically definite lesions of a relatively insignificant nature were found. They consisted in a perivascular fibrosis with some muscle destruction about small arteries and, less frequently, veins, situated especially in the wall of the right ventricle (Fig. 1). More rarely there was a small area of fibrosis at the base of a papillary muscle or within the wall of the left ventricle. The auricles showed no abnormality. Most of these lesions were healed and must have occurred soon after the first doses were given. They appeared somewhat more pronounced in the group which received desiccated thyroid. In the heart of one animal that died of thyroxin intoxication 9 days after the initial dose there were very acute small focal necroses about the wall of veins in the right ventricle. These lesions evidently occurred a few hours before death.

Altogether the effect of stimulation of the heart by thyroid products appeared to be injurious to the myocardium following the first doses, but no lesions indicated that this effect was progressive. The very definite relation of muscle injury and repair to blood vessels, both arteries and veins, suggests the possibility of a mechanical basis of origin, depending upon alterations in the circulation of blood rather than a direct toxic action of the thyroid products upon the muscle cells. This view is strengthened by the characteristics of the lesion in certain instances in which there does not appear to be a primary necrosis of muscle cells, but an interstitial fibrosis about the vessels.

The Effect of Chloroform on the Myocardium Stimulated by Desiccated Thyroid or Thyroxin.

The above experiments formed the basis and control for the following study to determine the susceptibility of the myocardium of animals stimulated by thyroid products to injury by known toxic substances, notably chloroform. While the toxic action of chloroform on the heart is well known, light anesthesia for an hour on 2 or 3 successive days in normal rabbits does not produce necrosis of the myocardial cells, as was shown in ten control animals. It has been found, however, that a similar procedure with rabbits that have received desiccated thyroid or thyroxin for several days until their heart rate becomes accelerated to about 300 beats per minute will usually produce necrosis of the myocardium, especially of the left ventricular wall, and often the destruction is very severe and diffuse. It is necessary in order to obtain more uniform results that chloroform be administered by inhalation, for while the total quantity of the drug absorbed by this method is much less than was introduced for comparison by subcutaneous injections in olive oil or by stomach, the effects on the heart muscle are very much greater. This is to be explained, I think, by a natural assumption that a greater concentration of chloroform during the period of inhalation acts upon the heart than is the case at any time when the drug is absorbed through the other routes. Following chloroform inhalation in these animals there is usually no central necrosis of the liver, even when a very extensive necrosis of myocardium results, whereas after the administration of 0.2 cc. of chloroform per kilo in equal quantity of olive oil subcutaneously the animal will die usually within 24 hours, with extreme central necrosis of the liver and little or no necrosis of heart muscle. Ordinarily the liver is more readily injured by chloroform inhalation than is the cardiac muscle and the only explanation of the absence of central hepatic necrosis in the experiments on hyperthyroidism animals is that the cardiac muscle is rendered, by the administration of thyroid products, more easily injured than the normal heart, and that an insufficient quantity of chloroform is inhaled to produce toxic necrosis of the liver. By the method of inhalation employed the animal is never deeply anesthetized. If

deep anesthesia is obtained the rabbits rarely live 12 hours and apparently die of cardiac failure. The amount of chloroform actually received by inhalation is regulated largely by reflex inhibition of respiration which is quite easily induced in these animals and is attended by inhibition of the heart. If the animals are not sufficiently anesthetized to lose this reflex, they are safe from immediate untoward effects.

In the experiments to test the effect of chloroform on the myocardium, young rabbits weighing from 1,800 to 2,500 gm. were used. They received a diet of oats, hay, carrots, and spinach. Desiccated thyroid gland was given to three animals in 1 gm. daily doses for periods of 12, 16, and 17 days respectively, then chloroform by inhalation for 1 hour. They were killed 24 to 48 hours later by a blow on the head, and tissues fixed immediately. A sample protocol follows:

Rabbit 3.—Brown; young adult male.

Aug.	19, 1920.	Weight 3,325 gm.;	pulse 208.				
"	20.	" 3,281 "	" 206.				
"	21.	" 3,157 "	" 180.				
"	23.	" 3,245 "		thyroid	1	gm.	
"	24.	" 3,248 "	pulse 224;	"	1	"	
"	25.	" 3,195 "	" 206;	"	1	"	
"	26.	" 3,145 "	" 224;	"	1	"	
"	27.	" 3,041 "	" 254;	"	1	"	
"	28.		" 262;	"	1	"	
"	30.	Weight 2,785 gm.;	" 224;	"	1	"	
"	31.	" 2,800 "	" 278;	"	1	"	
Sept.	1.	" 2,758 "	" 314;	"	1	"	
"	2.	" 2,792 "	" 300;	"	1	"	

10.45 a.m. Light chloroform anesthesia for 1 hour. Pulse rate during anesthesia: 11 a.m. 374; 11.25 a.m. 364; 11.45 a.m. 356. Got up immediately after chloroform was stopped.

Sept. 3, 9 a.m. Weight 2,675 gm.; pulse 316. Pulse weak and absolutely irregular. Thyroid 1 gm. 4 p.m. Pulse 448; irregular.

Sept. 4, 11 a.m. Pulse 400. Very irregular and weak. Looks sick. Killed by blow on head.

Autopsy.—Lungs clear, pink. Heart large, dilated; weight 10 gm. Walls thick and edematous. Both ventricular walls streaked and mottled with large opaque yellowish gray areas most marked in interventricular septum and right ventricle though present throughout both ventricles. Liver, central fatty change. Adrenals small.

Microscopic Examination.—Diffuse acute necrosis of myocardium of both ventricles.

This animal showed a much more extensive necrosis than the other two in which microscopic acute focal necroses of muscle cells were present in each ventricle. So extensive a necrosis has not been duplicated in rabbits receiving thyroxin.

Because of the complexity and lack of uniformity of desiccated thyroid gland, crystalline thyroxin was used in the subsequent experiments, twelve in number. Doses of 0.5 to 1 mg. were injected into an ear vein, usually every 2nd day, chloroform inhalation for an hour was given later, from 2 to 23 days after the initial injection. In some instances chloroform inhalation was repeated on the 2nd or 3rd day after the first inhalation. The animals were killed 24 to 72 hours after treatment with chloroform. The results of these experiments are given in Table I.

Rabbit 4.—Young, gray, three-fourths grown male.

Mar. 1, 1921. Weight 1,840 gm.; pulse 230; thyroxin 1 mg.
" 2. " 1,695 " " 290; diarrhea.
" 3. " 1,610 " " 320; "

9.50 a.m. Light chloroform inhalation, 1 hour. Pulse during inhalation: 10 a.m. 296; 10.10 a.m. 296; 10.20 a.m. 288; 10.30 a.m. 304; 10.40 a.m. 280; 10.50 a.m. 275. Got up immediately after chloroform was removed.

Mar. 4. Weight 1,550 gm.; pulse 348. No evident diarrhea. Pulse regular, strong. Thyroxin 0.5 mg.

Mar. 5. Weight 1,480 gm.; pulse 360. Stools soft.

Mar. 6. Weight 1,450 gm.; pulse 348; thyroxin 0.5 mg.

Mar. 7. Weight 1,370 gm.; pulse 364. Light chloroform inhalation for 1 hour. Pulse during inhalation: 10 a.m. 376; 10.10 a.m. 360; 10.20 a.m. 384; 10.30 a.m. 340; 10.40 a.m. 368; 10.50 a.m. 356; 11 a.m. 380. Very lightly under; came out immediately.

Mar. 8. Weight 1,230 gm.; pulse 340. Heart beat weak but regular, even after struggling for 2 minutes. There seem to be two rhythms, palpable over heart, one more rapid at base. Electrocardiogram shows regular beat. 3.30 p.m. Killed by blow on head.

Autopsy.—Ventricles collapsed and fibrillating. Large opaque yellowish patches in wall of left ventricle. Small opaque dots in right. Lungs, emphysematous; fresh focal hemorrhages posteriorly in both lower lobes. (Both emphysema and fresh peribronchial hemorrhages occur in these animals as a result of sudden blow on head. In one instance rupture of the liver was found.)

Microscopic Examination.—Diffuse necrosis of left and focal necrosis of right ventricle were found. No necrosis of liver.

TABLE I.

Intravenous Injections of Crystalline Thyroxin Followed by Chloroform Inhalation.

Animal No.	Weight.	Amount of thyroxin.	Duration of treatment.	Period of chloroform inhalation.	Total length of experiment.	Results.
	gm.	*mg.*	*days*		*days*	
5	2,652	4.5	6	1 hr.	10	Killed 4 days after chloroform. Diffuse necrosis in left ventricle, focal in right.
6	2,195	7	6	1 "	7	Killed 3 days after chloroform. Diffuse necrosis in left ventricle.
7	2,493	6	11	1 " each 2 days.	12	Killed 1 day after last chloroform inhalation. No necrosis.
8	1,695	3	4	1 "	4	Died 6 hrs. after chloroform. No necrosis.
9	1,432	3	4	1 "	4	Died 8 hrs. after chloroform. No necrosis.
10	2,447	3	4	1 "	5	Killed 1 day after chloroform. Focal necrosis.
11	2,500	6	10	1 "	11	Died 18 hrs. after chloroform. Focal necrosis.
12	2,400	4	7	1 " each 2 days.	10	Killed 3 days after first chloroform inhalation, 1 day after last. Diffuse necrosis.
4	1,840	2	2	1 " " 2 "	6	Killed 1 day after last chloroform inhalation, 5 days after first. Diffuse necrosis.
13	1,815	3	8	1 "	9	Killed 1 day after chloroform. Focal necrosis.
14	2,190	4	8	1 "	9	Killed 1 day after chloroform. Focal necrosis.
15	1,800	3	9	1 "	10	Killed 1 day after chloroform. Diffuse necrosis.

In none of these animals was there evidence of sufficient chloroform poisoning to have caused death had they not been killed, and undoubtedly all would have recovered, although in some instances

as much as one-fourth of the left ventricular myocardium appeared to have been destroyed. The lesions, however, were uniform, not progressive, and healing ensued with surprising rapidity, consequently if the rabbit survived the first 24 hours after chloroform the worst effects were safely over.

In five of the twelve rabbits that received thyroxin and chloroform there were large macroscopic areas of necrosis in the left ventricle and focal microscopic areas in the right; in three were microscopic foci in both left and right ventricles, and in three no necrosis was found, two of these having died within 24 hours after chloroform. No lesions were found in the auricles in any of these animals.

Characteristics of the Lesion.

In order to avoid the possibility of confusing any lesions produced experimentally with a preexisting scarring of the myocardium (a condition, however, not seen in any of the controls), the rabbits have been killed at short intervals after the inhalation of chloroform. In this way it has been possible to study the acute changes and to follow the process of repair. It is of interest that in no instance has simple fatty degeneration without necrosis been found in the animals which received thyroid products either alone or with chloroform. Fatty degeneration of the myocardium is the commonest pathological change noted in hearts from cases of exophthalmic goiter, yet it was not present in the two cases of hyperthyroidism reported by the writer except as an incident in necrosis. So in the experimental lesions cells in the neighborhood of necrotic areas contain an excess of fat, but it is quite evident that fatty degeneration did not precede the necrosis. In one instance there were patches of fatty degeneration with relatively little necrosis corresponding in distribution to necrotic areas in other hearts. This change obviously indicated injury but not sufficient to result in cellular death. The necrotic cells contain fractured bundles of fibrillæ, granules of various kinds, and often irregular transverse bands of hyaline material. The dead cells within a few hours become surrounded and invaded by large mononuclear phagocytes which are found in very great numbers within 48 hours (Fig. 2), incorporating particles of the disintegrating cells. At this time the fibroblasts are also extremely active, proliferat-

ing *in situ* and wandering in from the adventitia of neighboring blood vessels. Mitotic figures are present in great numbers. Polymorphonuclear leucocytes play no part in the lesion.

At the end of 72 hours (Figs. 3 and 4) small lesions have been replaced by young fibroblasts and in another 24 hours the lesions are practically completely healed by a loose scar tissue in which no wandering cells remain. Considerable edema accompanies the lesions but no hemorrhages or vascular changes have been observed. There is to be detected often a very definite perivascular distribution of necrosis, especially in the right ventricle. It may be found in connection with both arteries and veins. No evidence whatever of regeneration of cardiac muscle cells has been observed. In one instance fusion of mononuclear phagocytes occurred, forming giant cells similar in appearance to those of the Aschoff bodies appearing in myocarditis of rheumatic fever.

DISCUSSION.

It has been demonstrated by postmortem studies upon the human heart that a severe terminal acute necrosis of the cardiac muscle may occur in cases of hyperthyroidism dying of myocardial exhaustion without the usual evidences of profound intoxication with which this lesion is commonly associated. It seemed likely that these hearts, overstimulated by disease of the thyroid gland and laboring in a condition bordering on exhaustion, were in a state of greater susceptibility to injury by toxic substances such as may have resulted from a relatively mild terminal infection, or other sources, which under ordinary circumstances would not have materially injured the myocardium.

In the foregoing experiments a study has been made of the demonstrable effects of large doses of dried thyroid gland and of thyroxin upon the myocardium of rabbits. In brief it has been shown that rabbits receiving daily 1 gm. of thyroid gland or 1 mg. of crystalline thyroxin intravenously every 2nd day exhibit a marked increase in pulse rate, more forceful action of the heart, loss in body weight, increasing irritability, and frequently diarrhea and falling out of hair. Animals subjected to this treatment alone and killed at periods of 2 to 3 weeks may show relatively slight though definite lesions in

the myocardium, notably perivascular necrosis or fibrosis in the wall of the right ventricle, focal necrosis or fibrosis in papillary muscles of the left ventricle, and, more rarely, scattered small focal necroses within the myocardium elsewhere. These lesions evidently occur early in the treatment, for at the time examinations were made they were for the most part healed, and no evidence of progression under continued administration of thyroid products was observed.

The second series of experiments to test the susceptibility of the myocardium of similarly treated animals to injury by chloroform has yielded more striking results; that is to say, rabbits subjected to the feeding of 1 gm. of desiccated thyroid daily or receiving 1 mg. of crystalline thyroxin every 2nd day until their pulse rate reaches about 300 per minute, show in the majority of instances, following one or two light anesthesias from inhalation of chloroform vapor for 1 hour, a widespread necrosis of myocardium, sufficient in one instance to cause severe disturbances of cardiac function.

These results seem to support the view that a heart stimulated to an abnormal activity by products of the thyroid gland may be more readily injured than the normal heart; and they suggest a possible explanation for the severer degrees of cardiac disease in association with hyperthyroidism. One may well imagine that in the early stages of hyperthyroidism, when the heart is overstimulated by products of the diseased thyroid, acute infections or intoxications which would leave no significant impress on the normal person may be permanently injurious to the heart thus affected and with repetition of the injury the cardiac condition may become progressively worse.

The evidence does not indicate that products of the thyroid alone could be entirely responsible for the cardiac lesions which may occasionally occur in man in association with hyperthyroidism.

The immediate cause for the increased susceptibility to injury which these hearts exhibit is speculative. It occurs to us that in the state of hyperthyroidism and in the experimental condition following the administering of thyroid products, the nutritional reserve of the myocardium may be diminished, especially glycogen. Hearts of experimental animals in which cardiac necrosis has been produced show no glycogen microscopically, but whether a diminution in the amount of stored glycogen would predispose to injury is uncertain.

SUMMARY.

An experimental study upon the demonstrable effects of large doses of dried thyroid gland and thyroxin upon the myocardium of rabbits has been made. Animals under such treatment show characteristic clinical symptoms with definite, although relatively slight, myocardial lesions. Similarly treated animals which have, in addition, been subjected to chloroform anesthesia show more striking, widespread myocardial necrosis.

These experiments indicate very distinctly that chloroform as an anesthetic in cases of hyperthyroidism is apt to be exceptionally detrimental to the myocardium, and should be avoided.

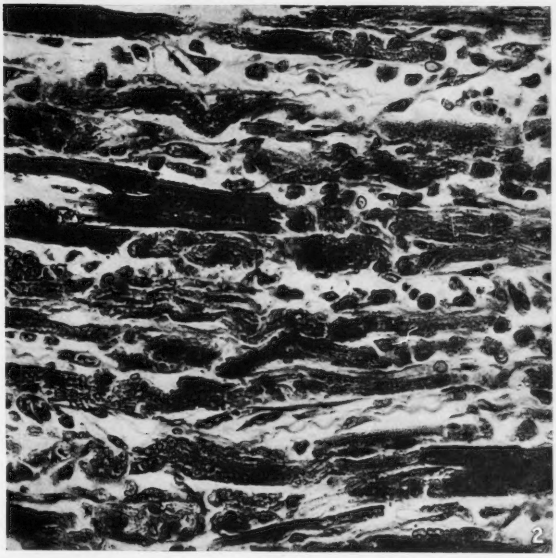
EXPLANATION OF PLATE 33.

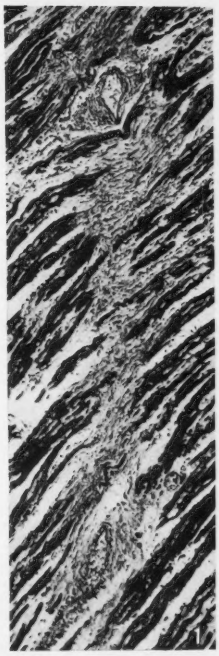
FIG. 1. Perivascular fibrosis in right ventricle following intravenous injection of thyroxin.

FIG. 2. Acute necrosis; 48 hours. Desiccated thyroid plus chloroform.

FIG. 3. Acute necrosis; 72 hours. Showing fibroblastic proliferation. Thyroxin plus chloroform.

FIG. 4. Acute necrosis; 72 hours. Thyroxin plus chloroform.





(Goodpasture: Myocardial necrosis.)